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Abstract

Title of Thesis: The Role of Dopamine in Normal Rodent Motor Cortex: Physiological Effects and Structural Correlates

Patrick William Awenowicz, Doctor of Philosophy, 1999

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Dopamine (DA) has been implicated in the cortical pathophysiology of several neurological disorders, including schizophrenia and Parkinson's Disease. Until recently, motor areas of the neocortex were thought to receive only sparse DA innervation. It is now known that the motor cortex of rodents and primates are densely innervated by DA, but its detailed circuitry and role in motor cortex function remain unclear.

Immunohistochemistry and in situ hybridization were used to determine the laminar distribution and morphology of neurons that contain the protein and mRNA for D1a, D2, and D5 receptors. Numerous pyramidal shaped neuronal somata in layers II-VI of rodent motor cortex, were immunoreactive for D1a, D2, and D5 receptors, and sparse non-pyramidal shaped neurons in layers V-VI were immunoreactive for the D1a receptor. Quantitative analysis revealed that all

three receptor subtypes were expressed by neurons with distinct laminar distributions.

Double label immunohistochemistry was used to determine if DARPP-32, a phosphoprotein that acts as part of the D1 receptor signal transduction cascade, co-localized D1a, D2, or D5 receptors in motor cortex neurons. DARPP-32 was co-localized with D1a and D2 receptors in pyramidal shaped neurons in layers V-VI, and with D5 receptors in neurons of deep layer VI.

Tract tracing and immunohistochemical techniques were used to determine if pyramidal tract neurons (PTNs), output neurons from the motor cortex to the spinal cord, possess D1a, D2, or D5 receptors. All three receptor subtypes were found in identified PTNs.

Locally applied DA induces both inhibitory and excitatory responses in the neocortex. Electrophysiological techniques were employed to determine the effects of iontophoretically applied DA on the spontaneous activity (SA) of PTNs, the receptors that mediate these effects, and DA's effects on glutamate induced excitation of PTNs. Locally applied DA inhibited the SA of PTNs, and this DA induced inhibition was blocked by both the D1 selective antagonist (SCH23390) and the D2 selective antagonist (eticlopride). Glutamate induced excitation of SA was reversed by DA.

These findings indicate that DA may differentially modulate numerous neurons in the motor cortex and may have profound effects on motor cortex activity, through its influence on PTNs.

The Role of Dopamine in Normal Rodent Motor Cortex: Physiological Effects and Structural Correlates

by

Patrick William Awenowicz

Dissertation Thesis submitted to the Faculty of the

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"It's closing time, it's time to go to the places that we'll be from"

-Semisonic

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INTRODUCTION

Motor cortex

The motor area of the mammalian cerebral cortex is located within the agranular frontal cortex (Vogt and Vogt, 1919; Zilles, 1990). It is a complex structure in primates made up of functionally distinct regions, including the primary motor (MI), the supplementary motor (SMA) (Penfield and Welch, 1951) and the premotor (PM) areas (Dum and Strick, 1991; for alternative classifications see Wise et al., 1991). The cytoarchitecture of these motor areas is distinct from other cortical regions in several ways. Most of the neocortex exhibits six cortical layers, distinguished by differences in cell size, cell packing density, and fiber orientation. The motor cortex has only five layers because it lacks a granule cell layer IV (Ramon y Cajal, 1909-1911). The presence of unusually large pyramidal neurons in layer V, further distinguishes the motor areas (Ramon y Cajal, 1909-1911). The largest of these neurons are named Betz cells, and compose a subpopulation of the pyramidal tract neurons (PTNs). The PTNs receive multi-modal information about ongoing movements (Fromm et al., 1984) and relay commands from the motor cortex to the spinal cord. They originate from widespread areas of the sensorimotor cortex, but in virtually all mammals, the MI gives rise to more of the PTNs than other cortical areas (Toyoshima and Sakai, 1982; Dum and Strick, 1991). The axons of PTNs descend in the internal capsule, then to the cerebral peduncle, and then to the

dorsal lateral funiculus of the spinal cord forming the pyramidal tract, also known as the corticospinal tract (Phillips and Porter, 1977). Fibers are topographically arranged only above the level of the cerbral peduncle (Nathan and Smith, 1955; Bernhard and Woolsey, 1956). Most axons of the PTNs end by contacting interneurons in the spinal cord. In primates however, some axons of MI PTNs have monosynaptic connections with spinal motor neurons that innervate the distal upper extremities (Bernhard et al., 1953; Bernhard and Bohm, 1954; Preston and Whitlock, 1961). Through these monosynaptic connections the pyramidal tract is the most direct path of communication between the motor cortex and muscles.

The motor cortex is involved in the control of voluntary movement. Numerous lesion studies in primates have indicated that damage to the motor cortex or pyramidal tract results in an impairment of fine movements of the hand and digits (Buxton and Goodman, 1967; Lawrence and Kuypers, 1968; Castro, 1972; Woolsey et al., 1972). Electrophysiological studies have shown that the primary motor cortex is directly linked to the execution of movement, as there is a close correlation between MI neuronal firing and the electromyelograph activity recorded from muscles during contraction (DeLong, 1972; Anderson and Horak, 1985). The role of MI in fine motor control, such as skilled hand movements, is mediated through the pyramidal tract.

The SMA, which lies rostromedial to the primary motor cortex, is thought to be involved in the preparation for movement and sequencing of complex

motor tasks. Lesions to the SMA result in complex motor dysfunctions, including severe akinesia (Freund, 1987), and the inability to sequence complex motor tasks (Shima and Tanji, 1998). Electrophysiological studies show that SMA neuronal activity is tightly coupled with movement preparation (Tanji et al., 1980). Neurons in the SMA are active well in advance of movements (Alexander et al., 1990; Lang et al., 1991). Additionally, positron emission topography (PET) studies show increased SMA activity prior to movement, or as the subject imagines making the movement (Roland, 1987). Therefore, complex sequential movements and the preparatory phase of movement appear to involve the SMA. The PM area lies rostral to the primary motor cortex and lateral to the SMA. The role of the PM area is less clear, but it may be involved in the preparation phase of sensory triggered movements (Passingham, 1987).

The primate motor cortex is distinguished from the rodent motor cortex by its relatively larger representative size, especially of the SMA, and the presence of functionally distinct motor areas, the SMA and PM. The cytoarchitecture and primary functions of rodent motor cortex however, are similar to that observed in primates. For example, the motor cortex of rodents is characterized by the absence of a granule cell layer IV and abnormally large pyramidal neurons in layer V (Zilles, 1990). As in primates, a portion of the large layer V neurons form the pyramidal tract whose axons descend directly to the spinal cord. The pathway differs somewhat in that the tract lies dorsally in the cervical spinal regions (Paxinos and Watson, 1986), and it is unlikely that any axons terminate

directly on spinal cord motor neurons (Valverde, 1966; although see Elger, 1977). Motor cortex lesions cause similar deficits of motor function in rats and primates. Selective damage to the rodent motor cortex results in specific motor dysfunctions including, an inability to generate independent manipulatory movements of the digits and wrists, to organize such movements into timed sequences, and to perform them rapidly (Bures and Bracha, 1990). Therefore, although phylogenetically more developed, the primate motor cortex shares common features with the rodent motor cortex.

Dopamine

The neurotransmitters dopamine (DA), norepinephrine (NE) and epinephrine are categorized as catecholamines because they all have a characteristic catechol group made up of two hydroxyl groups attached to an aromatic ring. Dopamine is synthesized by the conversion of the amino acid tyrosine, to 3,4-dehydroxyphenylalanine (DOPA), by the rate limiting enzyme tyrosine hydroxylase (TH). DOPA is then converted into DA by DOPA decarboxylase. Dopamine is stored in synaptic vesicles of axon terminals until it is released or enzymatically converted to NE by dopamine-β-hydroxylase (DBH) (for review see Mehler, 1992). Termination of action of DA occurs by re-uptake by the dopamine transporter (DAT) into the nerve terminal, where the enzyme, monamine oxidase, removes the amine group, or by simple diffusion away from the synapse (Sewell, 1999). Tyrosine hydroxylase has been used as an

immunohistochemical marker to determine the distribution of DA terminals within the central and peripheral nervous systems (Mehler, 1992).

Dopamine is widely distributed throughout the brain. Areas such as the basal ganglia, hypothalamus, hippocampus, and neocortex contain high levels of DA (Joyce et al., 1993). Various brain functions such as learning (Beninger, 1983), feeding behavior (Cooper, 1993), and motor control (Agid, 1991) are in part modulated by DA. The ubiquitous distribution and roles of DA accentuate the need for further investigation of this complicated neurotransmitter system.

The mesocortical dopamine system

The neocortex is richly innervated by dopaminergic input. The contributors to cortical DA innervation are the DA producing neurons located in the ventral tegmental area, substantia nigra pars compacta, and to a lesser extent, the retrorubral field (Gaspar et al., 1992). The neurons in these midbrain structures give rise to axons that travel through the medial forebrain bundle to the neocortex (Lindvall and Bjorkland, 1987). Dopaminergic innervation to the cerebral cortex previously was thought to be sparse and restricted to only a few cortical areas. The advent of sensitive immunostaining techniques has revealed a widespread and heterogenous DA innervation pattern of the primate (Gaspar et al., 1989; Williams and Goldman-Rakic, 1993) and rodent neocortex (Berger et al., 1985; Descarries et al., 1987; Berger et al., 1991). The regional differences in density and distribution of the mesocortical DA system suggest

that DA has distinct influences over different cortical areas.

The motor cortex of humans and primates receives the densest DA innervation of all cortical regions (Gaspar et al., 1989; Williams and Goldman-Rakic, 1993), suggesting an important role for DA in motor cortex function. The primate motor cortex displays a bi-laminar distribution pattern of DA innervation, with the highest density of axons found in layers I-II and V-VI (Gaspar et al., 1989; Williams and Goldman-Rakic, 1993). The rodent motor cortex is also innervated by dopaminergic projections, albeit with fewer DA terminals than that in primates (Berger et al., 1991). The pattern of DA innervation in rodent motor cortex is similar to that of primates in that it is densest in layers I and V-VI (Berger et al., 1985; Descarries et al., 1987). The functional role of the dense DA projection to the motor cortex has yet to be determined.

Dopamine's effects are mediated in the cortex, as well as in other regions of the brain, through a family of receptors that are coupled to guanine nucleotide regulatory proteins (G-proteins). These G-protein coupled receptors are activated by the binding of a transmitter, which results in an intracellular cascade of events. These G-protein coupled receptors are structurally characterized by having seven transmembrane domains and three intracellular loops (Sibley et al., 1993). Five subtypes of DA receptors have been identified (Civelli et al., 1993; Gingrich and Caron, 1993). They are categorized as D1 or D2 like, based on different pharmacological and biochemical properties (Kebabian and Calne, 1979; Seeman and VanTol, 1994). The D1 family includes D1a and D5

receptors. Dopamine binding to D1 like receptors activates the enzyme adenylyl cyclase, which subsequently increases intracellular levels of cyclic 3',5' - adenosine monophosphate (cAMP) (Kebabian and Calne, 1979; Seeman and VanTol, 1994). In turn, cAMP phosphorylates dopamine-and-cAMP- regulated-phosphoprotein (DARPP-32) (Hemmings et al., 1984). DARPP-32 is a potent inhibitor of protein phosphatase-1, and thereby blocks the dephosphorylation of certain phosphoproteins (Hemmings et al., 1984). D1a and D5 receptors have a small third cytoplasmic loop and a long C terminus. Overall, there is 50% homology of sequence between D1a and D5 receptors, but the level of homology is increased to about 80% in the transmembrane domains (Sibley et al., 1993). The D2 family includes D2, D3, and D4 receptors. Dopamine binding to D2 like receptors inhibits the enzyme adenylyl cyclase, and also may be linked to other second messenger systems including activation of K+ channels, inhibition of Ca2+ channels, and phosphatidylinositol turnover (Neve et al., 1989; Albert et al., 1990; Seeman and VanTol 1994). D2 like receptors have a large third cytoplasmic loop and a short C terminus. There is an overall homology of 52% between D2 and D3 receptors, but this increases to 75% homology in the transmembrane domains (Sibley et al., 1993). The D2 receptor shares an overall homology of 41% and 39% with D3 and D4 receptors, respectively. The level of homology increases to about 56% for both D3 and D4 receptors within the transmembrane domains (Sibley et al., 1993). The varied structures and signaling pathways of the receptor subtypes may provide a

mechanism for DA to differentially modulate neuronal activity.

Dopamine receptors are widespread and display a heterogeneous distribution similar to that of DA axons throughout the rodent and primate neocortex. The D1 and D2 receptor families have been identified in motor areas of the neocortex, and details of their individual distribution patterns are being elucidated (Goldman-Rakic et al., 1990; Huntley et al., 1992; Joyce et al., 1993; Sawaguchi and Goldman-Rakic, 1993). In both primate and rodent motor cortex D1 and D2 like receptors display distinct laminar distribution patterns (Joyce et al., 1993). Moreover, the different subfamilies of receptors appear to be expressed in distinct populations of cortical neurons (Gaspar et al., 1995; Bergson et al., 1995b).

The precise role of DA in motor cortex is not well understood. Its potential influence in modulating cortical activity is reflected by the observation that neurons in virtually all cortical layers respond to DA (Sawaguchi et al., 1986a). Electrophysiological studies have demonstrated that locally applied DA has variable effects on cortical neuronal activity. Dopamine induces both inhibitory and excitatory responses in neurons of the prefrontal cortex of anesthetized animals, but inhibitory responses predominate (Bernardi et al., 1982; Sawaguchi et al., 1986a). Likewise, iontophoresis of DA during active voluntary movements inhibits task-related activity of some neurons and facilitates it in others of the monkey motor and prefrontal cortices (Sawaguchi et al., 1986b; Matsumura et al., 1990). Here again, inhibitory responses predominate. More information is

needed to determine how DA modulates specific populations of cortical neurons and through them, the overall output of the motor cortex.

While DA's role in normal cortical function remains uncertain, the mesocortical DA system has been implicated in the pathophysiology of several neurological disorders, including Parkinson's disease (PD) and schizophrenia (Gaspar et al., 1989; Williams and Goldman-Rakic, 1993). Animal models of DA depletion have been used extensively to understand the manifestations of PD. Neurotoxic lesions are used to selectively damage DA producing neurons in the midbrain and result in significant depletion of DA in the basal ganglia and motor cortex. Dopamine depleted rodents exhibit impaired motor behavior such as deficiencies in skilled motor tasks that require complex temporal sequencing (Whishaw et al., 1986; Salamone et al., 1990) and diminished accuracy and rate of skilled movements (Sabol et al., 1985; Whishaw et al., 1986). These motor deficits are similar to those observed after motor cortex lesions in rodents (Bures and Bracha, 1990), suggesting that DA is involved in mediating complex temporal sequencing of movements and execution of skilled movements in the rodent motor cortex.

Parkinson's disease in humans is characterized by a progressive degeneration of the midbrain dopaminergic neurons, which results in a gradual loss of DA innervation to many of its target areas. Traditionally, the symptomology of PD has been attributed to the disruption of the dopaminergic nigro-striatal pathway (Bernheimer et al., 1973; Hornykiewicz, 1982). However,

recent findings from post mortem brains of PD patients show that DA is markedly and selectively depleted in motor areas of the cortex (Gaspar et al., 1991). This cortical DA loss may be a contributing factor to some of the motor impairments of PD.

The hallmarks of PD symptomology are bradykinesia, rigidity and tremor. In addition, the patient's ability to perform a complex series of movements is disrupted and movement reaction times are increased (Marsden, 1989). Basal ganglia function has long been associated with these aspects of motor control, and the dopaminergic nigrostriatal pathway has been implicated in their modulation (Whishaw et al., 1986). However, the motor cortex is involved in some of these same aspects of motor control and its contribution is difficult to distinguish from that of the basal ganglia (Whishaw et al., 1986). Furthermore, it is unlikely that the drastic reduction in DA innervation of the motor cortex has no relevant functional consequences. Indeed, physiological and functional imaging studies show that motor cortex function is disrupted in PD. Positron emission tomography (PET) imaging studies have shown that patients with PD exhibit reduced activity in the PM and SMA during skilled hand movement, in comparison to controls (Playford et al., 1993; Rascol et al., 1997). Similarly, single photon emission computed tomography (SPECT) studies show alterations in vascular perfusion of the MI, SMA, and somatosensory cortex during sequential finger-thumb tasks in PD patients as compared to controls (Rascol et al., 1993). Additionally, transcutaneous magnetic stimulation of the motor cortex

has revealed abnormal responses in the motor cortex of PD patients (Priori et al., 1994). All of these findings indicate that DA depletion induces a disruption of cortical function in PD and accentuate the need for further studies to determine the function of DA in the normal motor cortex, as well as its role in the pathophysiology of PD.

Specific aims and hypotheses

The goal of this dissertation was to elucidate the dopaminergic circuitry and its functional role in the normal rodent motor cortex. The dense DA innervation to the frontal cortex, its ubiquitous effects on cortical neurons and its involvement in cortical dysfunction suggest that its influence over normal motor cortex function is profound.

The first **specific aim** of this dissertation was to determine the distribution and morphology of neurons that contain DA receptor subtypes. We hypothesized that distinct populations of neurons would express different DA receptors and that receptor subtypes would be distributed differentially throughout the cortical laminae. The mechanisms by which DA modulates cortical activity are not well understood. The effects of DA on target neurons are determined in part by the type of receptor that is activated. Identification of receptor subtypes on distinct populations of neurons will aid in determining DA's influence on the cortical circuitry. Immunohistochemical and *in situ* hybridization techniques were used to identify neurons that contain D1a, D2, and D5 receptor

subtype proteins and the mRNA for expression of these proteins. Histological analysis was done to determine the laminar distribution of the receptor proteins and the morphological classification of neurons that contain them.

The second **specific aim** was to determine if identified pyramidal tract neurons possess DA receptors. We hypothesized that PTNs would express a specific subtype(s) of DA receptors. Signals from the motor cortex that control skilled movements are relayed through PTNs to the spinal cord. Dopamine may modulate the activity of these neurons through direct input or indirectly through the local circuitry. Tract tracing techniques to identify PTNs were coupled with fluorescent immunohistochemical stains to identify D1a, D2, and D5 receptors in these neurons.

The third **specific aim** was to determine the physiological effects of DA on PTN activity and the receptor subtypes that mediate these effects. We hypothesized that locally applied DA acting through specific receptors would alter the spontaneous activity of PTNs and the glutamate induced excitation of PTNs in the rodent motor cortex. Modulation of PTNs may have profound effects on the output of the motor cortex, as they provide a balance of inhibitory and excitatory influences over synergistic muscles, which is critical for coordination of skilled motor tasks. Electrophysiological techniques were used to identify PTNs. Dopamine was applied iontophoretically in conjunction with DA antagonists, to determine DA's effects on the spontaneous activity of PTNs and to identify the receptors that mediate the response. Similar experiments were

done to determine the effect of DA on glutamate induced excitation. The results of these experiments are expected to provide information about which neuronal populations may be vulnerable to DA influences, as well as how DA may effect identified cortical neurons. These findings will aid in the understanding of the role of DA in normal motor cortex function.

SPECIFIC AIM 1 AND 2

The first **specific aim** of this dissertation was to determine the distribution and morphology of neurons that contain DA receptor subtypes.

The second **specific aim** was to determine if identified pyramidal tract neurons possess DA receptors.

Introduction

Dopamine receptor subtypes in the motor cortex

Each of the five known DA receptor subtypes have been positively identified in the motor cortex (Lidow et al., 1989, 1991; Huntley et al., 1992). For the most part, the distributions of DA receptors have been characterized with autoradiographic receptor binding techniques. Selective ligands that bind to either the D1 or D2 family of receptors are currently available, but those that bind to specific receptor subfamilies have not been identified. Binding of the D1 selective ligand ^{3}[H]SCH23390 indicates that the DA D1 receptor family has a distinct laminar distribution in the rodent frontal cortex. These receptors are present predominantly in the infragranular layers V and VI, but sparse D1 ligand binding appears in layers II-III (Boyson et al., 1986; Richfield et al., 1989; Mansour et al., 1990, 1992; Vincent et al., 1993). The distribution of D2 receptors in the rodent frontal cortex is somewhat more controversial. Studies using the D2 selective ligands ^{3}[H]-raclopride or N-*p*-aminophenethyl-spiperone report that D2 receptors are found predominantly in the deep cortical layers

(Mansour et al., 1990; Vincent et al., 1993). Other studies using the D2 selective ligand ^{3}[H]-spiroperidol, report that D2 receptors are found throughout layers I-VI of rodent frontal cortex, with the highest density in the superficial layers I-II (Richfield et al., 1989). More precise methods such as *in situ* hybridization and immunohistochemistry have reconciled some of the conflicting reports and have revealed greater details of the distribution patterns of DA receptor subfamilies.

In situ hybridization uses known cDNA strands to identify the presence of mRNA for specific proteins in a cell. Results of *in situ* hybridization studies for the D1a receptor are similar to those of ligand binding studies for the D1 family in rodent neocortex. This technique demonstrates that the highest density of neurons containing D1a mRNA are in layers II, V and VI, whereas fewer neurons containing D1a mRNA are in the middle layers III-IV (Mansour et al., 1992; Gaspar et al., 1995; LeMoine and Gaspar, 1998). A limited number of studies have focused on D5 receptor distribution in rodent cortex, whereas a more extensive examination has been done in primate cortex. *In situ* hybridization studies have shown that D5 mRNA is sparse throughout the rodent neocortex, including motor areas (Sunahara et al., 1991; Tiberi et al., 1991), but further details are not known. In primates, D5 mRNA is distributed throughout layers II-VI of the neocortex, with the highest levels found in layer V (Huntley et al., 1992; Meador-Woodruff et al., 1996; Lidow et al., 1998).

Recently, antibodies to the different receptor proteins, including D1a, D2, and D5, were generated (Huang et al., 1992; Ariano et al., 1993; Levey et al.,

1993; Bergson et al., 1995a). The production of these antibodies has made it possible to apply immunohistochemical techniques to study the distribution of the receptor proteins. Varying results have been reported for D1a immunoreactivity in the rodent neocortex. Some studies find D1a receptor labeling to be very extensive and distributed throughout layers II-VI (Huang et al., 1992; Ariano et al., 1993), while others demonstrate very light D1a labeling (Levey et al., 1993). Different sensitivities of the antibodies used in these studies may account for these conflicting findings. Immunohistochemical studies for D1a receptors in primate neocortex report that labeled neurons are located in layers II-VI (Smiley et al., 1994; Bergson et al., 1995b), with the highest densities in layers II, III, and V (Bergson et al., 1995b). Therefore, D1a receptor distribution in rodents shares some similarities to that in primates.

An immunohistochemical analysis of the D5 receptor protein in primates showed that D5-immunoreactive (ir) neurons in prefrontal and premotor cortex are spread throughout layers II-VI, with the highest proportion of D5-ir neurons located in layers II, III, and V (Bergson et al., 1995b). In this same study, the somata and apical dendrites of pyramidal neurons are intensely labeled for the D5 receptor (Bergson et al., 1995b). Because D5 receptor protein expression and the presence of D5 mRNA are dense in cortical layer V of primates, we were interested in determining whether similar patterns of D5 distribution exist in the rodent.

Similar to receptor binding studies of D2 distribution, *in situ* hybridization

experiments reveal contradictory findings in rodents. Some groups report that D2 receptor mRNA is found in neurons of layers II-VI, with increased levels in layers I-III (Mansour et al., 1990). Others however, report a more restricted pattern, with D2 mRNA present predominantly in layer V (Gaspar et al., 1995; LeMoine and Gaspar, 1998). In primate frontal cortex, D2 mRNA appears in neurons throughout layers II-VI, with the highest levels in layer V (Huntley et al., 1992; Lidow et al., 1998). Antibodies to D2 receptors have been used to determine the distribution of the receptor protein (McVittie et al., 1991; Levey et al., 1993). Low levels of D2 immunoreactivity occur in the rodent neocortex (Levey et al., 1993). In general, D2 receptor expression is much lower than that of D1, in both primate and rodent cortex (Joyce et al., 1993).

The phosphoprotein DARPP-32 is part of the DA D1 receptor signal transduction cascade (Ouimet, 1988). Therefore, DARPP-32 has been used in the past as a putative marker for the D1a receptor. Immunohistochemical studies using antibodies directed against DARPP-32 have shown that the protein is located in neurons of layers V and VI of rodent frontal cortex (Ouimet, 1988). This distribution overlaps with that of D1a, D2, and D5 receptors, suggesting that DARPP-32 might be co-localized with D1a, D2, or D5 receptors in the rodent neocortex. A recent report that only half of the striatal neurons that are immunopositive for DARPP-32, are also immunopositive for the D1a receptor (Langley et al., 1997), indicates that DARPP-32 may co-localize with another DA receptor subtype. Our studies explore this possibility in the rodent neocortex.

The rodent cortex provides a valuable animal model for study of DA in normal cortical function and of disrupted cortical function related to perturbations of the dopaminergic system such as that in schizophrenia and Parkinson's disease. However, a comprehensive examination of DA receptor distribution is lacking. This portion of the study has three objectives: 1) To determine the laminar distribution and morphological characteristics of neurons in the rodent motor cortex that express D1a, D2, or D5 receptors, or DARPP-32; 2) To determine the extent of co-localization of D1a, D2, and D5 receptors with DARPP-32 in rodent motor cortex; 3) To determine the laminar distribution of D1a, D2, and D5 mRNA in rodent motor cortex.

Dopamine receptor subtypes on pyramidal tract neurons

In the motor cortex, pyramidal neurons are distributed throughout all cortical laminae. These neurons utilize the excitatory neurotransmitter glutamate and compose approximately two thirds of all cortical neurons (Sloper et al., 1979). Their axons form a complex network of horizontal intracortical connections and link cortical regions through cortico-cortical connections (Gatter and Powell, 1978; DeFelipe et al., 1986; Ghosh et al., 1988). Furthermore, pyramidal neurons provide virtually all of the outbound signals from the cortex.

Ultrastructural studies that show pyramidal neurons are the primary targets of DA afferents in the primate and rodent neocortex (Van Eden et al., 1987; Segula et al., 1988; Verney et al., 1990; Smiley and Goldman-Rakic,

1993; Krimer et al., 1997). There are however, limited numbers of non-pyramidal neurons that are also synaptic targets (Smiley and Goldman-Rakic, 1993; Porter, 1995; Sesack et al., 1995). Dopaminergic axon terminals synapse with the spines and shafts of spiny dendrites in rat, monkey, and human neocortex (Van Eden et al., 1987; Segula et al., 1988; Goldman-Rakic et al., 1989; Verney et al., 1990; Smiley and Goldman-Rakic, 1993; Krimer et al., 1997). These post synaptic profiles are presumably the dendrites of pyramidal neurons, because most spines in the neocortex belong to pyramidal neurons (Feldman 1984). Dopamine terminals are often observed forming a symmetric synapse with a dendritic spine in close association with an unidentified terminal forming an asymmetrical synapse on the same spine (Goldman-Rakic et al., 1989; Verney et al., 1990). This arrangement has been termed a 'triad' and may be the morphological correlate of the physiological interactions observed between the dopaminergic and glutamatergic modulation of cortical neurons.

The mRNAs for D1a and D2 receptors have been identified in a few specific populations of pyramidal neurons in rodent frontal cortex. Corticostriatal and corticocortical neurons contain both D1a and D2 mRNA while corticothalamic neurons contain the mRNA only for D1 receptors (Gaspar et al., 1995). It has not yet been determined if these neurons express the receptor proteins. The potential for distinct populations of cortical neurons to express one or more receptor subtypes suggests that DA's modulatory effects are diverse not only within a cortical region but even within a specific population of neurons.

While these three populations of cortical projection neurons, identified as containing DA receptor mRNA, are involved in some aspects of motor control, their roles and the consequences of DA modulation of their activity, relative to voluntary movements are difficult to assess. We were interested in the relationship between DA afferents and PTNs. These neurons are important in translating motor cortex activity into voluntary movement because of their direct axonal projections to the spinal cord, and their direct and/or indirect connections with spinal cord motor neurons (Valverde, 1966). Given the fact that pyramidal neurons are the primary targets of DA afferents, and that a dense population of pyramidal neurons in layer V possess D1a, D2, or D5 receptors, it seemed likely that PTNs would possess one or more of these receptor subtypes. If this were the case, then it would appear that DA's modulation of cortical activity was important in skilled voluntary movements. Immunohistochemical staining for D1a, D2, and D5 receptor subtypes was coupled with the identification of PTNs by retrograde neuronal tracers to address this issue.

Methods

Immunohistochemistry

Tissue preparation

Adult male Sprague Dawley rats were used in these experiments. All procedures were done according to the NIH Guidelines for the Care and Use of Animals. Rats were deeply anesthetized with Ketamine HCl (60 mg/kg im) and

Xylazine (4 mg/kg im) and perfused intracardially with saline (0.9% NaCl) followed immediately by 4.0% paraformaldehyde in sodium phosphate buffer (PB: 0.1M sodium phosphate, pH 7.3). The brains were removed and cryoprotected overnight at 4°C in PB containing 20% sucrose. The brains were sectioned at -20°C with a freezing microtome in the coronal plane, and 40 µm thick sections through the motor areas of frontal cortex were collected (Paxinos and Watson, 1986). Alternating series of sections, including both hemispheres, were processed for localization of D1a, D2, D5, or DARPP-32, or for the co-localization of D1a, D2, or D5 with DARPP-32. Eight animals were used for identification of individual of markers and four animals were used for the co-localization of D1a, D2, or D5 with DARPP-32.

Immunohistochemistry for D1a, D2, D5, or DARPP-32

Free floating sections were incubated in PB (O.1M, pH 7.3) containing 0.3% H_2O_2 for 30 minutes. The sections were washed (3 X 10 min) in phosphate buffered saline (PBS: 0.1M sodium phosphate buffer, pH 7.3 with 2.0% NaCl) and then incubated in a blocking serum (PBS containing 3.0% normal horse serum, NHS) for 1 hour. The sections were then incubated with the appropriate primary antibodies (1:100 for rat anti-D1a monoclonal antibody, Research Biochemical International; 1:500 for rabbit anti-D1a polyclonal antibody, Dr. Marjorie Ariano; 1:1,000 for rabbit anti-D2 polyclonal antibody, Dr. Marjorie Ariano; 1:2,500 for rabbit anti-DARPP-32 polyclonal antibody, Chemicon; 1:200 for goat anti-D5 polyclonal antibody, Santa Cruz) in PBS containing 3% NHS

and 0.1% Triton X for 42-48 hrs at 4°C. The rat anti-D1a receptor antibody was a fusion protein generated from nt 1047-1339 encoding the C-terminal 97aa of the human D1a receptor and has been characterized previously (Levey et al., 1993). The rabbit anti-D1a receptor antibody was generated against peptide fragments 156-172, 226-235, and 284-292 of the rat D1a receptor and has been characterized previously (Ariano and Sibley, 1994). The rabbit anti-D2 receptor antibody was generated against peptide fragments 1-18, 19-36, 217-238, 253-270, 289-304, 348-365, and 331-444 of the rat D2 receptor and has been characterized previously (McVittie et al., 1991). The goat anti-D5 receptor antibody was generated against peptide fragment 455-472 of the rat D5 receptor and is non cross-reactive with other DA receptor subtypes.

Following washes (3 X 10 min) in PBS, the sections were incubated for 1 hour in PBS containing 3% NHS with a biotinylated secondary antibody (5 µl/ml goat anti-rat for D1a; 5 µl/ml goat anti-rabbit for D1a, D2, and DARPP-32; 5 µl/ml donkey anti-goat for D5, Vectastain Elite ABC kit). Controls for nonspecific binding of the secondary antibodies were performed by incubating sections in the absence of primary antibodies. A separate set of control sections were incubated with D1a and D5 receptor antibodies that were preadsorbed with the D1a receptor protein (RBI) or the peptide fragment used to generate the D5 receptor antibody (Santa Cruz). Sections were washed in PBS, incubated in PBS with avidin-biotin-peroxidase complex for 1 hour (ABC; Vectastain Elite ABC kit), washed in phosphate buffer, treated for 2-6 minutes with a DAB substrate kit for

peroxidase, and then rinsed in distilled H_20. Sections were mounted, dried, dehydrated, and covered. One series of alternating sections in each set was counterstained with CresylViolet to determine laminar borders and cytoarchitectonic boundaries.

Co-localization of D1a receptor protein with DARPP-32

Contrasting fluorescent markers were used to determine the co-localization of DARPP-32 with the D1a or D2 receptor. Sections were washed and rinsed with blocking serum as described above and then simultaneously incubated with primary antibodies for DARPP-32 and D1a for 42-48 hrs at 4°C. Following washes (3 X 10 min), sections were incubated sequentially in PBS containing 3.0% NHS, with the appropriate secondary antibodies (anti-rat IgG conjugated to Cy3 to label D1a, Jackson Immunoresearch and anti-rabbit IgG conjugated to AMCA to label DARPP-32, 1:200, Jackson Immunoresearch) for 30-60 minutes each. The sections were washed (3 X 10 min), mounted with Vectashield mounting medium (Vector Laboratories), sealed, and stored at 4°C in the dark.

Controls for non-specific binding of the secondary antibodies were performed by incubating the sections in the absence of primary antibodies. Three separate controls were used to assess cross-reactivity of the antigen-antibody complex: 1) Sections were incubated with one primary antibody and reacted with both secondary antibodies, 2) Sections were incubated with both primary antibodies and reacted with only one secondary antibody, and 3)

Sections were incubated with one primary antibody and reacted with the inappropriate secondary antibody.

Co-localization of D1a, D2, and D5 receptor proteins with DARPP-32

Double immunostaining, using secondary antibodies conjugated to fluorescent and biotinylated markers, was used to determine the co-localization of DARPP-32 with the D1a, D2, and D5 receptor, as described above. Briefly, sections were simultaneously incubated in primary antibodies for DARPP-32 (rabbit) and D5, or DARPP-32 (mouse) and D1a or D2, washed in PBS, and then incubated sequentially in the appropriate secondary antibodies. DARPP-32 (rabbit) immunoreactivity was visualized using a peroxidase reaction with DAB as the chromagen, and D5 immunoreactivity was visualized with anti-goat IgG conjugated to Cy3. DARPP-32 (mouse) immunoreactivity was visualized with anti-mouse IgG conjugated to Cy3, while D1a and D2 immunoreactivity were visualized using a peroxidase reaction with DAB as the chromagen. The optimal order of processing secondary antibodies was determined to be as follows: the ABC reaction, the incubation in Cy3, and then the DAB reaction for 2-3 minutes. The sections were mounted with Vectashield mounting medium (Vector Laboratories), sealed, and stored at 4°C in the dark.

Controls were prepared as described for D1a/DARPP-32 co-localization.

Analysis

The laminar distribution of D1a, D2, D5, or DARPP-ir neurons within the primary motor cortex was analyzed histologically with a Nikon microphot light

microscope. The primary motor cortex was delineated by its cytoarchitectonic features (Zilles, 1990). This region is located over the genu of the corpus callosum and lateral to the cingulate cortex, within the lateral agranular frontal cortex (Donoghue and Wise, 1982). The morphological classification of neurons was possible when labeling extended sufficiently into the dendritic arbors of immunoreactive neurons. Pyramidal neurons were identified by their pyramidal shaped soma and prominent apical dendrites (Feldman, 1984). A limited number of interneurons were identified by their radial dendritic arbors and lack of an apical dendrite.

Digitized images were generated using a video camera mounted on a Nikon microphot light microscope, which was directly linked to a computer. Images were collected from an area of motor cortex 800 μm in width and extending from the pial surface to the white matter. The digitized images were collected and saved in an imaging program (Image Pro), and montaged in Adobe Photoshop, to reconstruct the motor areas in each section. The laminar borders were determined by Nissl stained adjacent sections and delineated in the digitized images. Automated cell counts of immunoreactive neurons were made with Image Pro to determine the laminar location of labeled neurons visible in a single focal plane. Manual cell counts of ir neurons from randomly selected digitized images were done to compare with the automated cell counts. This procedure was done to ensure that our criteria for automated selection of neurons was accurate. Analysis of the laminar distribution of each marker was

done in 12 montages from the primary motor cortex. Three sections, taken from each of four animals were used to generate the montages. The laminar distribution of receptors was expressed as the percent of total immunoreactive neurons per layer.

The co-localization of DA receptor proteins with DARPP-32 was done with a Nikon microphot fluorescent microscope by examining the area delineated as primary motor cortex for the presence of single and double labeled neurons. Immunoreactive neurons tagged with the secondary fluorochrome Cy3 were visualized through a filter with the excitation range 510-560 nm and barrier filter at 590 nm. Immunoreactive neurons tagged with the secondary fluorochrome AMCA were visualized through a filter with the excitation range 355-375 nm and barrier filter at 400 nm. Immunoreactive neurons tagged with the avidin-biotin complex and reacted with DAB were visualized with incandescent light. A total of 12 sections, from 4 animals, for each marker, were carefully examined for neurons that were double-labeled for D1a, D2, or D5 and DARPP-32.

In situ hybridization

Probes and stock solutions

The D1 oligonucleotide probe used in our studies, consisted of a 30-base synthetic oligonucleotide from 162-191 of the coding region. The D2 oligonucleotide probe used in our studies, consisted of three 45-base synthetic oligonucleotides from 28-72; 426-471; and 537-582 of the coding region. The D5

oligonucleotide probe used in our studies, consisted of two 24-base and one 18-base synthetic oligonucleotide from 132-156; 282-300 and 1109-1133 of the coding region. The randomer probe was a 36-base synthetic oligonucleotide. The probes were tailed with Biotin-16-UTP at the 3' end. All solutions used were treated with 0.01% DEPC (Diethyl Pyrocarbonate) and autoclaved or filtered sterile. The hybridization buffer (HyB) was comprised of 2X SSC, 1X Denhardt's solution, 50% Deionized formamide, 125 ug/ml yeast tRNA, and 10% dextran sulfate.

Tissue processing

Six adult rats were deeply anesthetized (Xylazine, 4 mg/kg im and Ketamine HCl, 60 mg/kg im) and perfused transcardially with filtered 4% paraformaldehyde. The tissue was harvested and cryoprotected by soaking overnight in 20% sucrose 0.1M sodium phosphate buffer. The brains were then frozen in liquid isopentane (-30^{0}C; 1 min). The brains were sectioned in the coronal plane, at -20°C with a cryostat and 20 µm thick sections through the motor areas of frontal cortex were collected (Paxinos and Watson, 1986). Alternating series of sections, including both hemispheres were processed for the localization of D1a, D2, and D5 receptor mRNA.

After one hour at room temperature, the sections were exposed to three distinct conditions: 1) Incubated with biotinylated probe (100 ng) in HyB with 100 ug/ml denatured salmon sperm overnight; 2) Incubated with non-biotinylated probe (100 ng) in HyB with 100 ug/ml denatured salmon sperm overnight; and 3)

Incubated with RNase A(200 ug/ml in 100 mM tris and 0.5 M NaCl) for 30 minutes prior to overnight incubation with biotinylated probe (100 ng) in HyB with 100 ug/ml denatured salmon sperm in HyB. The incubation was in a 37^{0}C humidified chamber. Included within each of the three treatment groups was a D1 probe, D2 probe, D5 probe, a randomer, and HyB alone.

The following treatments were applied to all sections. After overnight incubation the sections were washed with 2 X SSC for 15 minutes at room temperature, then a 15 min wash was done at 37^{0}C, followed by a stringency wash with 0.2X SSC for 15 min at 37^{0}C. Sections were then blocked with 0.5% BSA in 0.1M PBS for 30 min at room temperature, and then incubated with mouse-anti-biotin (1:100 primary antibody) in 0.2% BSA in 0.1 M PBS for one hour at room temperature. Following 2 X 10 minute washes in 0.1 M PBS with 0.5% Triton X the sections were then exposed to immunohistochemical processing. The sections were incubated for one hour in PBS containing 2% BSA with a biotinylated secondary antibody (5 µl/ml goat anti-mouse), then sections were washed in PBS and processed with the avidin-biotin-peroxidase complex for one hour (ABC; Vectastain Elite ABC kit), and visualized with a DAB substrate kit for peroxidase (2 - 6 minutes). The sections were rinsed in distilled H_20, dried over night, dehydrated in a series of alcohols and xylene, and coverslipped with DPX.

A total of 12 sections, from 4 animals, for each probe were analyzed using light microscopy to determine the laminar and regional distribution of D1a, D2,

and D5 receptor mRNA.

Fast Blue Injections

Animals were prepared for aseptic surgery in a designated area according to the NIH Guideline for the Care and Use of Animals. Rats were anesthestetized with Xylazine (2-4 mg/kg; im) and Ketamine HCl (60 mg/kg; im). Supplemental doses (Xylazine 1-2 mg/kg and Ketamine 30 mg/kg; im) were given as needed throughout the surgical procedure. Animals were monitored to ensure that they remained areflexive. Animals were placed in a stereotaxic apparatus. Body temperature was maintained by a thermoregulating heating pad. Ophthalmic ointment was applied to the eyes.

A local anesthetic, xylocaine (1 ml; 20 mg/ml), was injected subcutaneously at the base of the skull. A midline skin incision was made. A laminectomy was performed over the cervical region of the spinal cord to expose the corticospinal tract (Paxinos and Watson, 1986). A glass pipette (tip diameter 20 µm) mounted on an electrode carrier was filled with the retrograde flourescent tracer, Fast Blue (FB, 2.0% in saline), and inserted to a depth of 200-400 µm from the surface, into the corticospinal tract. FB was injected bilaterally into the corticospinal tract by regulated air pressure injection with a Picospritzer (40 psi; 50 msec). The pipette was withdrawn and the wound area was closed in layers, using absorbable sutures for muscle and connective tissue and nonabsorbable sutures for the skin. The animals were monitored during recovery and given postoperative analgesics as needed.

Immunohistochemistry coupled with FB

Eight days after the FB injection, rats were deeply anesthetized with Ketamine HCl (60 mg/kg; im) and Xylazine (4 mg/kg; im) and perfused intracardially with saline (0.9% NaCl) followed immediately by 4.0% paraformaldehyde in sodium phosphate buffer (PB: 0.1M sodium phosphate, pH 7.3). The brains were removed and cryoprotected overnight at 4°C in PB containing 20% sucrose. The brains were sectioned in the coronal plane, at -20°C with a freezing microtome and 40 µm thick coronal sections through the motor areas of frontal cortex (Paxinos and Watson, 1986) were collected. Alternating series of sections, including both hemispheres, were processed for immunohistochemical localization of D1a, D2, and D5 receptors.

Free floating sections were washed (3 X 10 min) in phosphate buffered saline (PBS: 0.1M sodium phosphate buffer, pH 7.3 with 2.0% NaCl) and then incubated in a blocking serum (PBS containing 3.0% normal horse serum, NHS) for one hour. Sections were then incubated with the appropriate primary antibodies (1:100 for rat anti-D1a monoclonal antibody, Research Biochemical International; 1:500 for rabbit anti-D1a polyclonal antibody, a gift from Dr. Marjorie Ariano; 1:1000 for rabbit anti-D2 polyclonal antibody, a gift from Dr. Marjorie Ariano; 1:200 for goat anti-D5 polyclonal antibody, Santa Cruz) in PBS containing 3% NHS and 0.1% Triton X for 42-48 hrs at 4°C. Subsequently, the sections were washed (3 X 10 min) in PBS with 3.0% NHS. Then incubated in PBS containing 3.0% NHS, with the appropriate secondary antibodies (anti-

rabbit IgG conjugated to Cy3 to label D1a and D2, 1:400, Jackson Immunoresearch or anti-goat IgG conjugated to Cy3 to label D5, 1:400, Jackson Immunoresearch) for 30-90 minutes. The sections were washed (3 X 10 min), mounted with Vectashield mounting medium (Vector Laboratories), sealed and stored at 4°C in the dark.

Analysis

The area delineated as primary motor cortex was carefully examined for the presence of single and double labeled neurons with a Nikon microphot fluorescent microscope. Immunoreactive neurons tagged with the secondary fluorochrome Cy3 were visualized through a filter with the excitation range 510-560 nm and barrier filter at 590 nm. Neurons labeled with the retrograde fluorescent tracer FB were visualized through a filter with the excitation range 355-375 nm and barrier filter at 400 nm. Sections were carefully examined for FB labeled neurons that were immunopositive for D1a, D2, or D5 receptors.

Results

Immunohistochemistry

D1a receptors

Sparse numbers of D1a-ir neurons were detected in the rodent motor cortex using the rat anti-D1a monoclonal antibody. In each 40 µm section, a mean of only six D1a-ir neurons were observed. The D1a containing neurons labeled with this probe were restricted to layers V-VI. Label was observed in the somata and throughout extensive portions of the dendritic arbors of these

neurons. The dendritic labeling made it possible to classify these neurons as multipolar. Figure 1 A is a montage of a section through layers V-VI of rodent motor cortex immunolabeled for D1a in which a single labeled neuron is visible. Figure 1 B shows the D1a-ir neuron in layer V at higher magnification.

Neurons immunoreactive for the rabbit anti-D1a polyclonal antibody were much more numerous than those for the rat anti-D1a monoclonal antibody. With this probe, D1a-ir neurons were observed throughout cortical layers II-VI. Figure 2 A shows a montage of digitized images from a section of rodent motor cortex extending from the pial surface to the white matter. The section was immunolabeled for D1a. Figure 2 B shows labeled neurons in a portion of layer VI at higher magnification. Automated analysis of the distribution of labeled neurons revealed that 23% of the total D1a-ir neurons were found in layers II-III, 33% were in layer V, and 44% were in layer VI (n=12961). Many neurons were difficult to classify morphologically because only very proximal, if any, portions of their dendrites contained label. However, some were identified as pyramidal neurons by their pyramidal shaped somata and prominent apical dendrites. Some non-pyramidal neurons were labeled for D1a receptors. These cells had extensive dendritic labeling and were restricted to layers V-VI, similar to the population labeled by the rat D1a monoclonal antibody.

D2 receptors

D2-ir neurons also were observed throughout layers II-VI of rodent motor cortex. Automated analysis of the distribution of labeled neurons revealed that

Figure 1

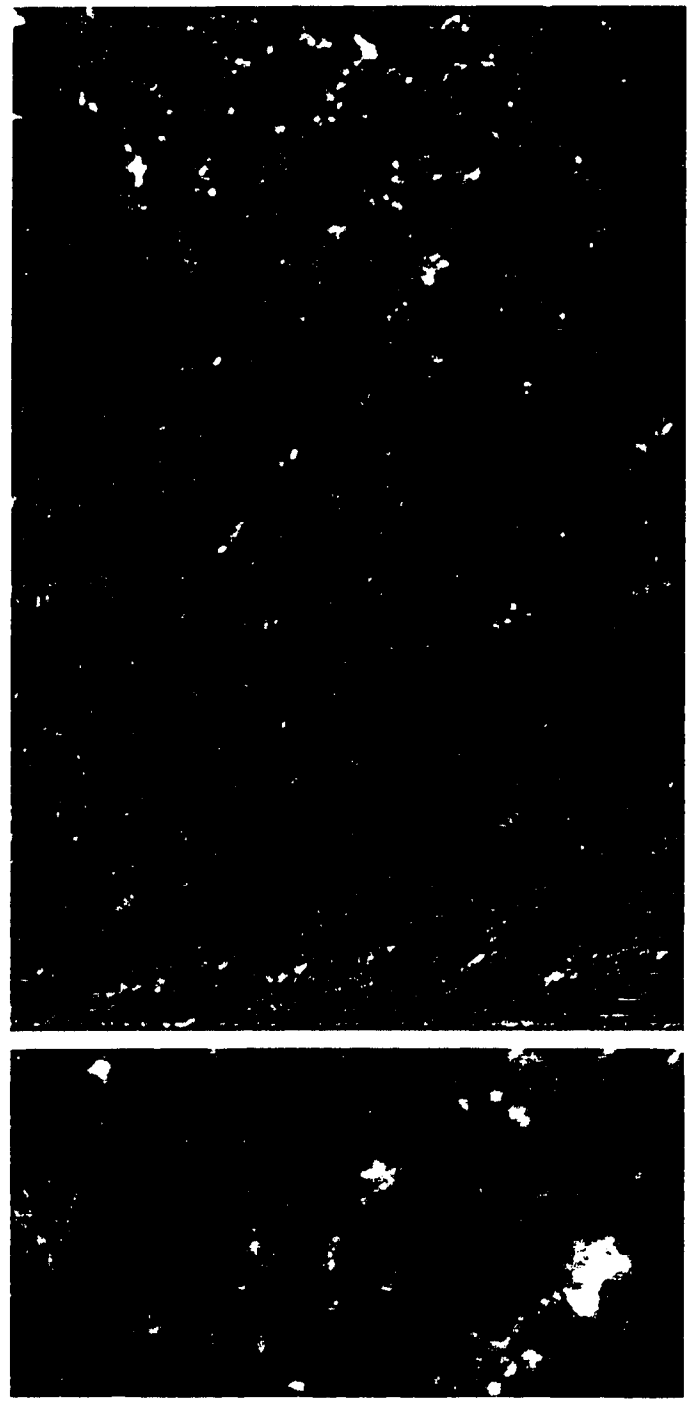


Figure 1. Montage of a section through the deep layers of rodent motor cortex, immunolabeled for the D1a receptor (rat anti-D1a). A) Montage which shows the location of a single non-pyramidal D1a-ir neuron in deep layer V (scale bar = 50 μm). B) The same neuron is shown at higher magnification to demonstrate the extensive dendritic labeling (scale bar =20 μm).

Figure 2

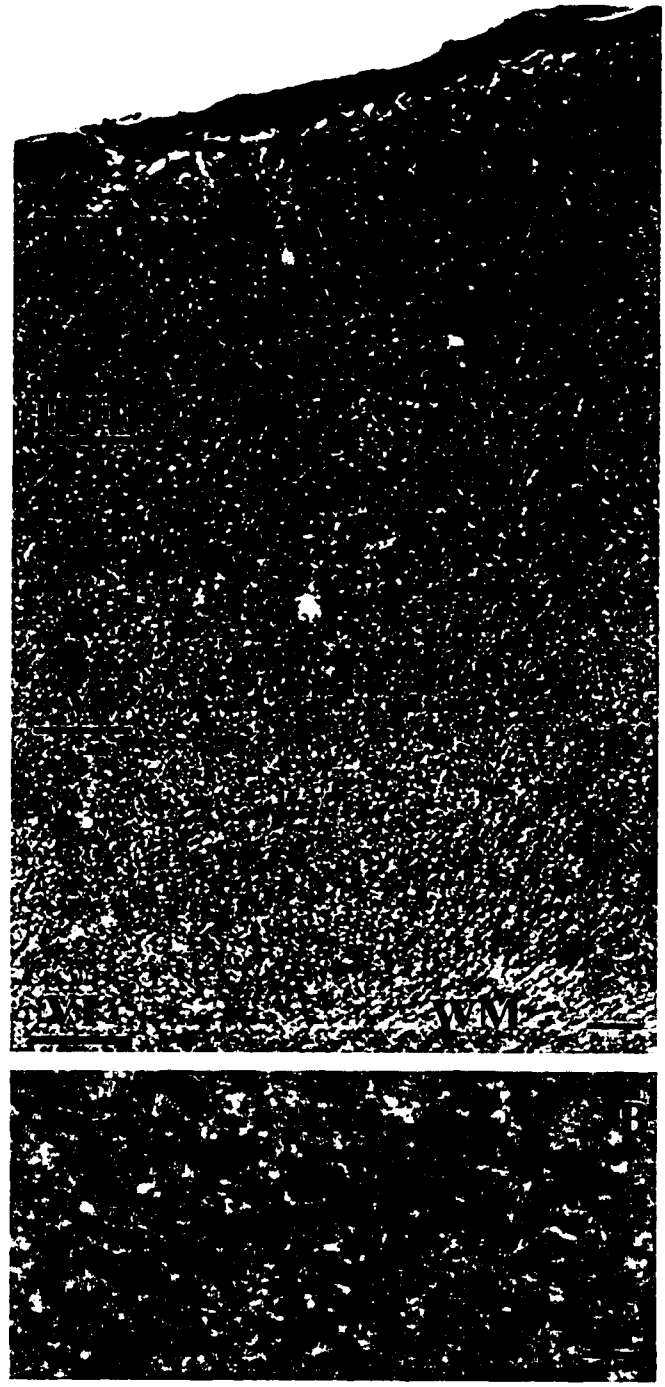


Figure 2. Montage of a section of rodent motor cortex immunolabeled for the D1a receptor (rabbit anti-D1a). A) Montage that extends from the pia to the white matter. Labeled neurons are visible in all cortical layers (scale bar = 50 μm). B) Higher magnification of D1a-ir neurons in layer VI, to show the density of labeled neurons (scale bar = 50 μm).

26% of the total D2-ir neurons were found in layers II-III, 35% were in layer V, and 37% were in layer VI (n=13199). D2 immunoreactivity was predominantly located in the somata of pyramidal shaped neurons, although some labeling was observed in the proximal apical dendrites. Most D2-ir neurons were identified as pyramidal neurons by the pyramidal shape of their somata and their prominent apical dendrite. Figure 3 A shows a montage of digitized images from a section of rodent motor cortex immunolabeled for D2. Labeled neurons are visible throughout the cortical layers. The higher magnification image in 3 B reveals somata that are pyramidal shaped with label extending into the proximal portions of an apical dendrite.

D5 receptors

Neurons that contained D5 receptors were observed in cortical layers II-VI. Analysis of the distribution of labeled neurons revealed that 15% of the total D5-ir neurons were found in layers II-III, 62% were in layer V, and 23% were in layer VI (n=4216). Within layer VI, D5-ir neurons were primarily restricted to the deep half of the layer. Similar to labeling for the other receptors, D5 immunoreactivity was located primarily in somata and proximal dendrites. Most of these labeled neurons were identified as pyramidal neurons by their pyramidal shaped somata and the prominent apical dendrite. Neurons in layer V however, exhibited dendritic labeling that extended into portions of dendrites reaching well into the superficial layers. Figure 4 A is a montage of digitized images immunolabeled for D5. Labeled neurons are dense in layer V, although visible in

Figure 3

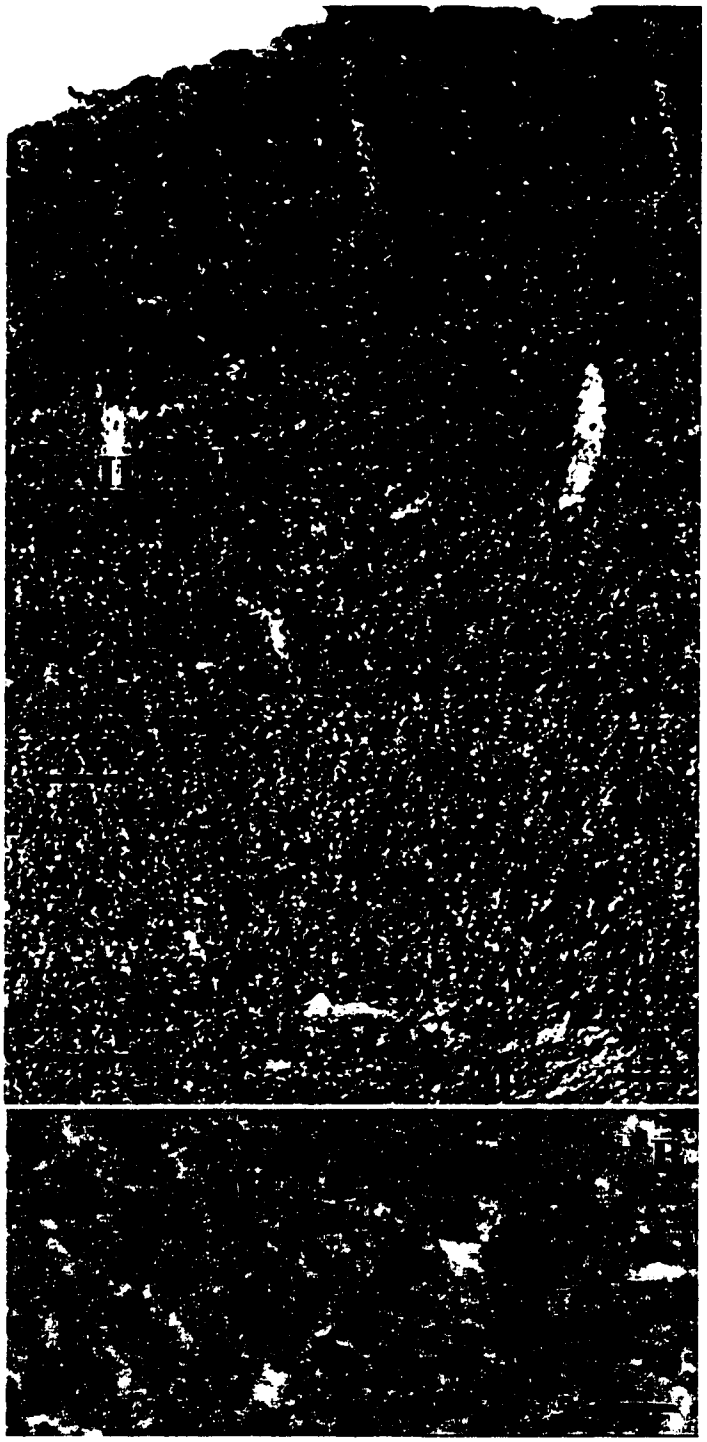


Figure 3. Montage of a section of rodent motor cortex immunolabeled for the D2 receptor. A) Montage which shows D2-ir neurons distributed through all cortical layers (scale bar = 50 μm). B) Higher magnification of D2-ir neurons in layer V from the same section as in A, to show the pyramidal morphology of some labeled neurons (scale bar = 30 μm).

Figure 4

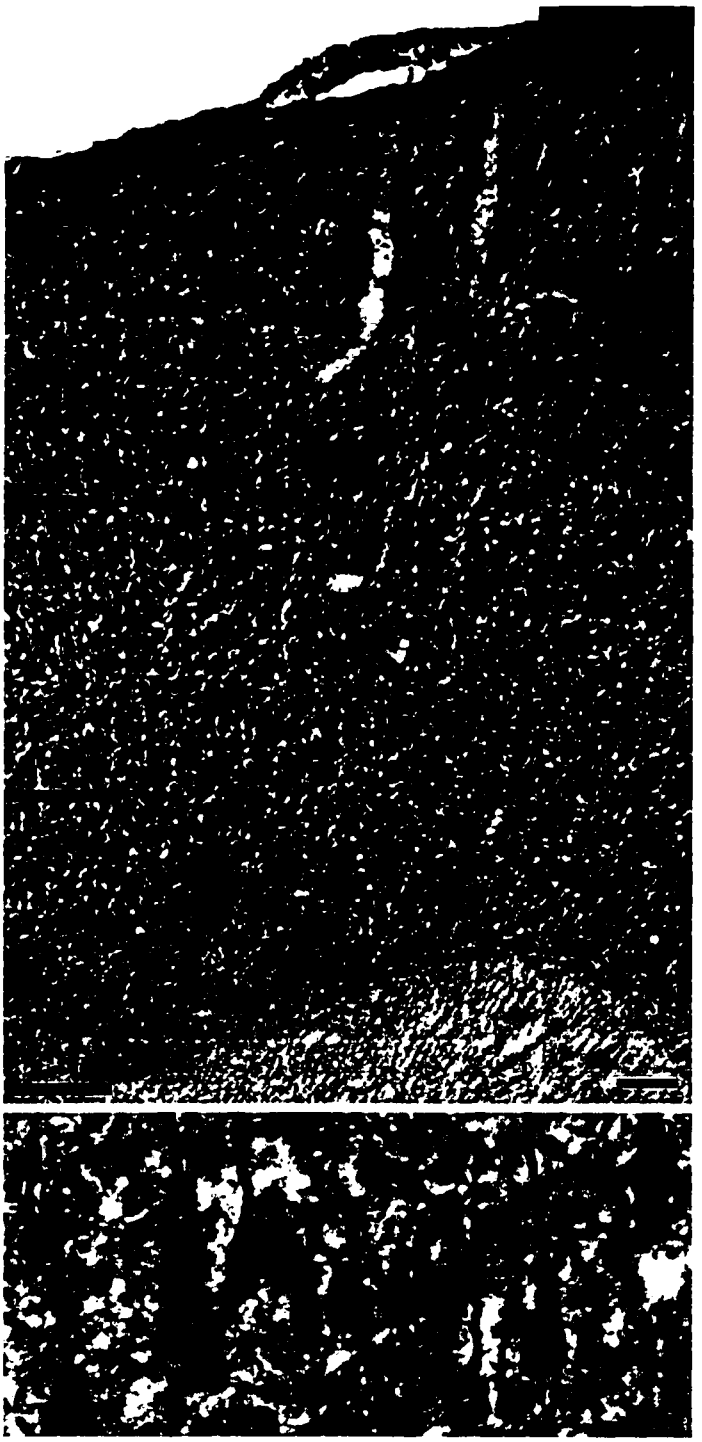


Figure 4. Montage of a section of rodent motor cortex immunolabeled for the D5 receptor. A) D5-ir neurons are located throughout layers II-VI, but are most dense in layer V (scale bar = 50 μm). B) Higher magnification of D5-ir neurons in layer V. Label extends into extensive portions of the primary apical dendrite (scale bar = 30 μm).

other areas. Labeled neurons in 4 B display distinct apical dendrites.

DARPP-32

Neurons that contained DARPP-32 were also numerous. DARPP-32-ir neurons were observed throughout layers II-VI of the rodent motor cortex. Five percent of all DARPP-32-ir neurons were found in layers II-III, 31% were in layer V, and 64% were in layer VI (n=7897). DARPP-32 immunoreactivity was predominantly observed in somata and proximal apical dendrites making it possible to identify nearly all DARPP-32-ir neurons as pyramidal neurons. This population of DARPP-32-ir neurons was distributed heterogeneously across layers II-VI. Figure 5 A shows a montage of digitized images immunolabeled for DARPP-32, with dense neuronal labeling in layers V-VI. The higher magnification in 5 B shows that some neurons have pyramidal shaped somata and label in proximal portions of apical dendrites. A small subpopulation of DARPP-32-ir neurons was observed and classified as non-pyramidal neurons. These neurons exhibited

extensive dendritic labeling and were predominantly multipolar in shape. Examples are shown in Figure 6 A-B. In each section, a mean of only eight DARPP-32-ir non-pyramidal neurons were observed. These neurons were all restricted to layers V and VI.

No detectable labeling was observed in any of the control sections.

Figure 7 summarizes the laminar distribution of D1a, D2, and D5 receptors and DARPP-32.

Figure 5

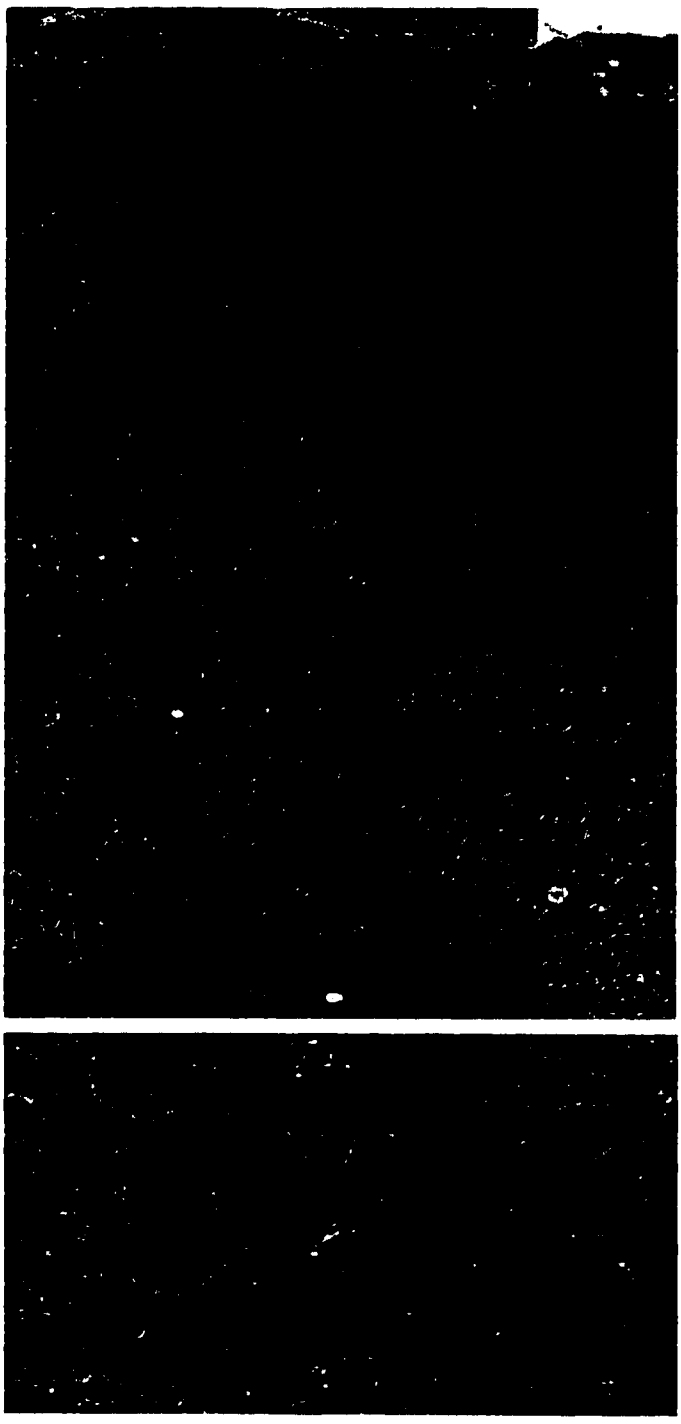


Figure 5. Montage of a section of rodent motor cortex immunolabeled for DARPP-32. A) DARPP-32-ir neurons are visible throughout layers II-VI, but are most dense in layer VI (scale bar = 50 μm). B) Higher magnification of DARPP-32-ir neurons in layer VI shows labeled neurons, some of which have pyramidal shaped somata and label in apical dendrites (scale bar = 30 μm).

Figure 6

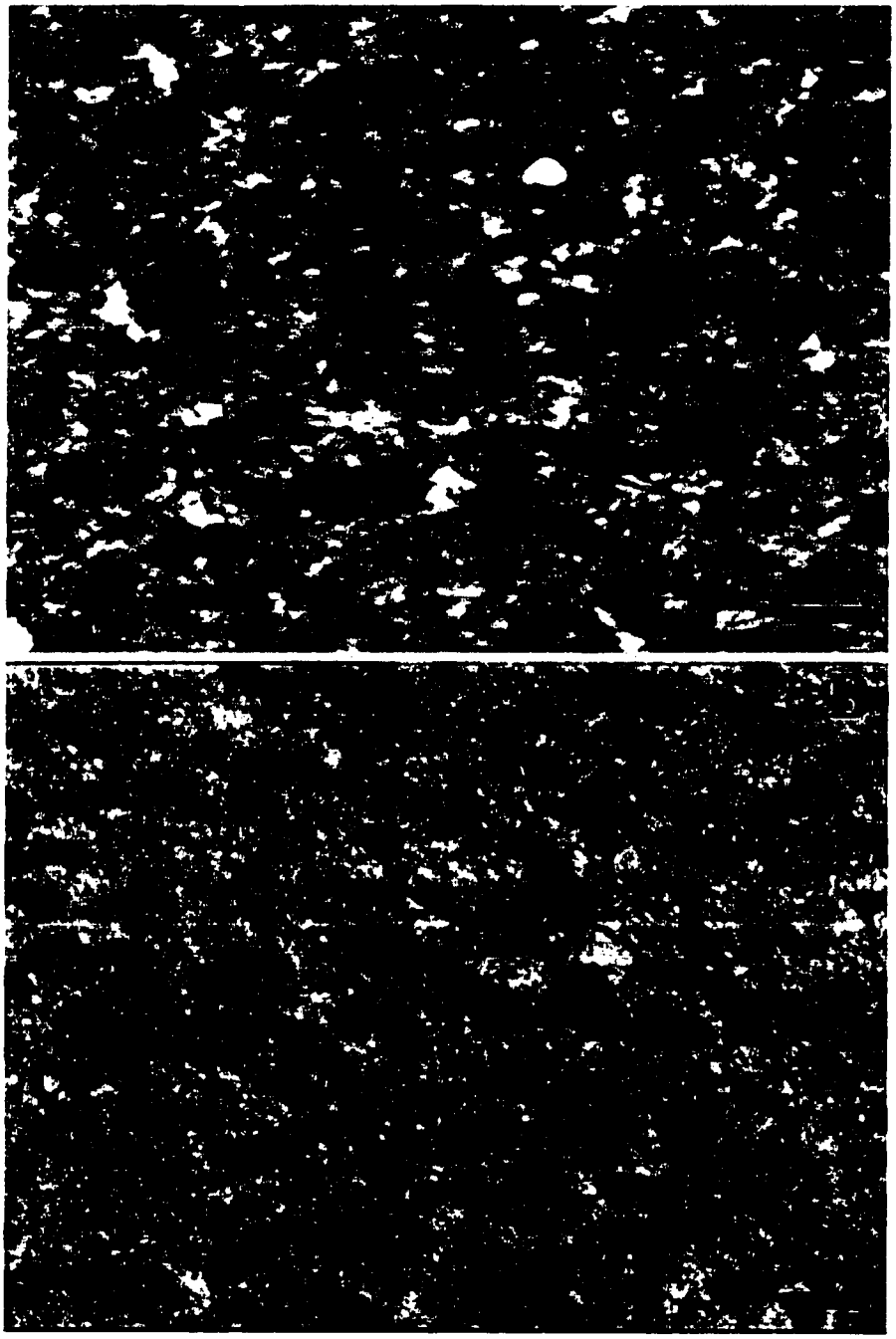


Figure 6. Digitized images of a section through the rodent motor cortex showing DARPP-ir non-pyramidal neurons. A) A non-pyramidal neuron immunoreactive for DARPP-32 in layer V. Extensive dendritic labeling is evident. B) A non-pyramidal neuron immunoreactive for DARPP-32 in layer VI. Extensive dendritic labeling is evident. (scale bar = 50 μm for A and B).

Figure 7.

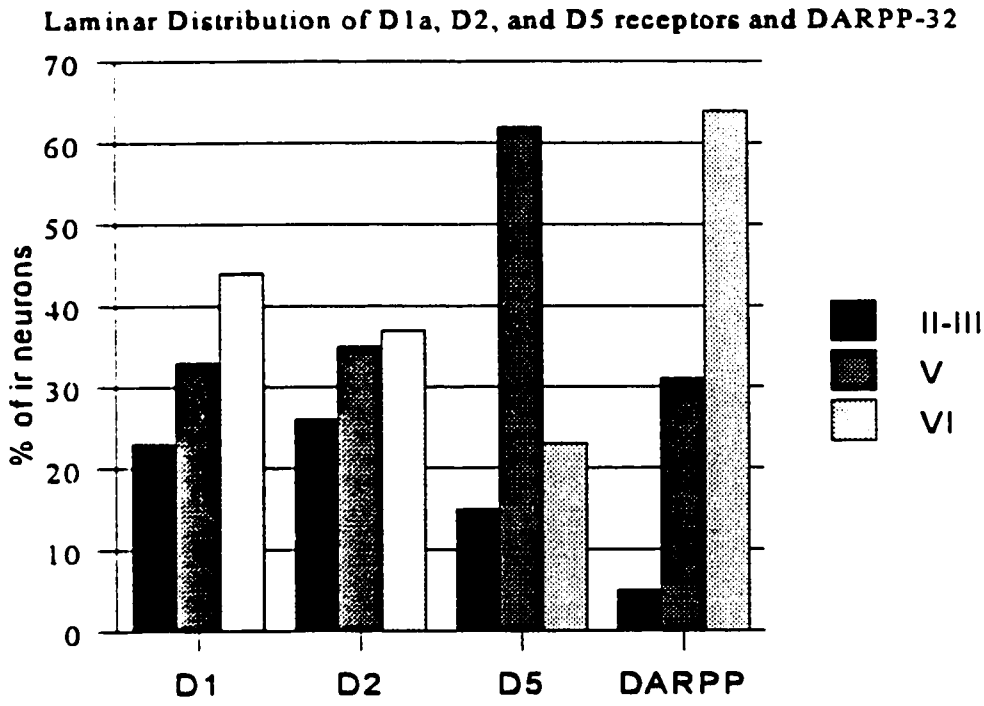


Figure 7. Histogram of the laminar distribution of D1a, D2, and D5 receptors, and DARPP-32 determined from automated counts of immunoreactive neuronal somata. The percent distribution was determined by the number of immunoreactive neurons in a layer of rodent motor cortex, divided by the total number of immunoreactive neurons across cortical laminae in the same section.

Co-localization of the receptor proteins with DARPP-32

Double labeling techniques revealed that DARPP-32 was co-localized with D1a receptors in the non-pyramidal neurons observed in layers V and VI. Figure 8 A-B shows double labeled layer VI non-pyramidal neurons, that contained extensive dendritic labeling. The entire population of these non-pyramidal D1a-ir neurons co-localized DARPP-32, while only approximately 75%

of the similar DARPP-32-ir non-pyramidal neurons co-localized the D1a receptor. Many other D1a-ir neurons in layers V-VI, that were observed with the anti-rabbit probe, also co-localized DARPP-32. Examples of such double labeled neurons are shown in Figure 8 C-D.

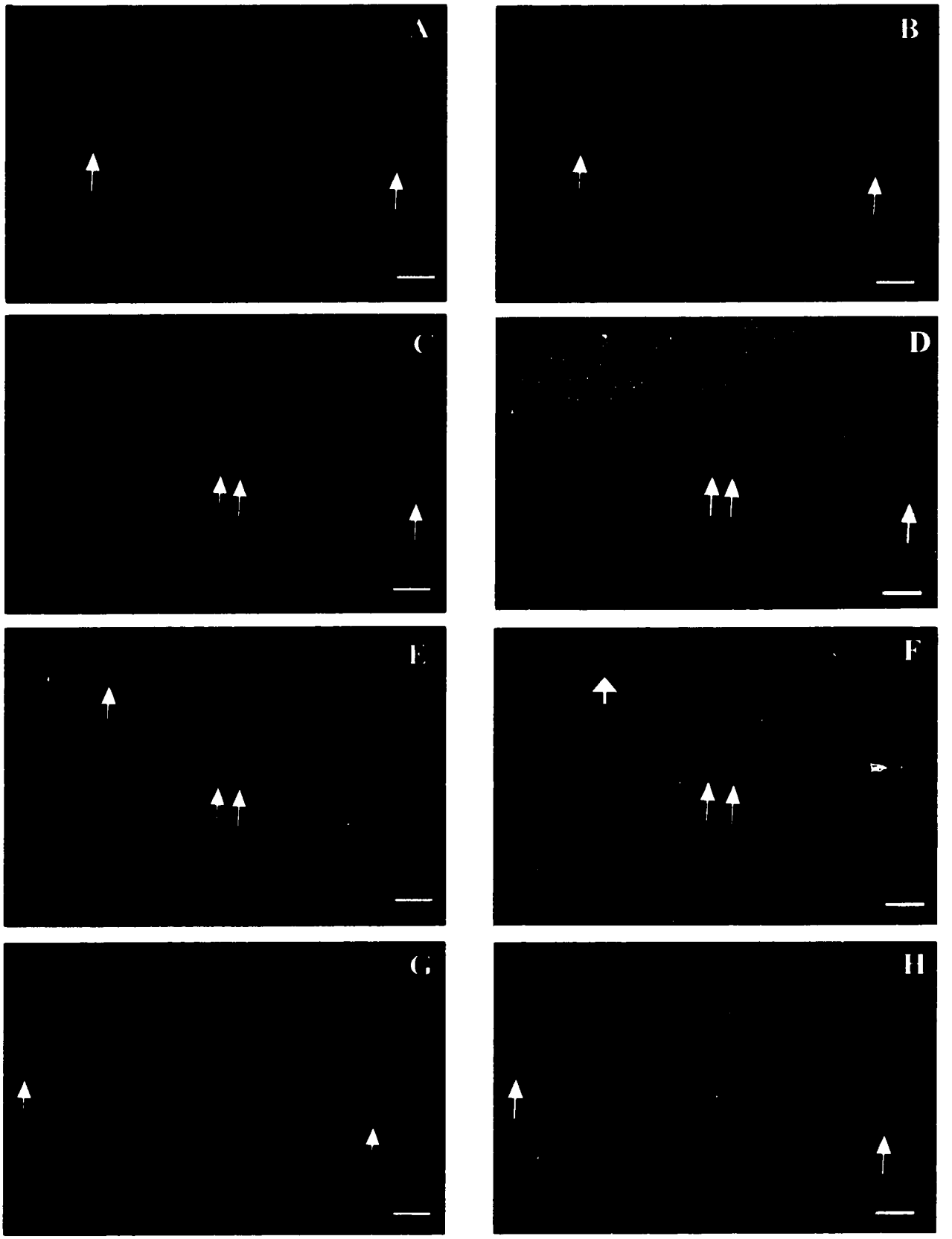
Double labeling revealed that DARPP-32 co-localized with D2 receptors in many neurons of layers V-VI. Examples of DARPP-32 and D2 receptor double labeled neurons are shown in Figure 8 E-F.

DARPP-32 also co-localized with D5 receptors but only in a subpopulation of layer VI neurons (Fig 8 G-H). These neurons were all located in the deep aspect of layer VI, forming a band of double labeled cells deep to the DARPP-ir only neurons and superficial to the D5-ir only neurons. While both D5 receptors and DARPP-32 were found in neurons of layers II-VI, they co-localized only in neurons of layer VI.

Omission of the primary antibodies did not result in detectable labeling. Incubation of sections with a single primary antibody and the inappropriate secondary antibody did not result in detectable labeling. Incubation in both primary antibodies with only one secondary antibody resulted in labeling of only the appropriate primary antibody. Sections incubated with a single primary antibody and both secondary antibodies, resulted in specific labeling of the primary antibody applied. Incubation of sections with preadsorbed antibodies resulted in no detectable labeling. Manual cell counts were similar to the automated counts.

Figure 8. Photomicrographs of neurons in rodent motor cortex that co-localize DARPP-32, and D1a, D2 or D5 receptors. A) Photomicrograph of two neurons in layer V that are D1a-ir (rat), visualized with the Cy-3 fluorochrome. Both had extensive dendritic label and were identified as non-pyramidal B) Photomicrograph of DARPP-32 labeled neurons in layer V, visualized with the AMCA fluorochrome. The same two neurons are labeled for D1a in A and B. C) Photomicrograph of neurons in layer VI that are DARPP-32-ir neurons labeled with Cy-3. D) Photomicrograph of the same section as in C, showing D1a-ir (anti-rabbit probe) neurons in layer VI, visualized with the chromagen DAB. Arrows indicate the same double labeled neurons. E) Photomicrograph of neurons in layer VI that are DARPP-32-ir, visualized with Cy-3. F) Photomicrograph of the same section as in E, showing D2-ir neurons in layer VI, labeled with the chromagen DAB. Arrows indicate double labeled neurons (scale bar = 100 μm for A - F). G) Photomicrograph of neurons in deep layer VI that are DARPP-32-ir, visualized with Cy-3. H) Photomicrograph of the same section as in G, showing D5-ir neurons in deep layer VI, visualized with the chromagen DAB. Arrows indicate the same double labeled neurons (scale bar = 200 μm for G - H).

Figure 8



In situ hybridization

Neuronal somata that contained mRNA for the D1a receptor were found in layers II-VI of rodent motor cortex. Figure 9 shows a montage of digitized images labeled for D1a mRNA. Their distribution pattern was similar to that of neurons containing the D1a receptor protein. Neuronal somata that contained mRNA for the D2 receptor were observed in layers II-VI of rodent motor cortex. Figure 10 shows a montage of digitized images labeled for D2 mRNA. The density of labeled neurons appeared to be highest in layers II-III. Very little D5 mRNA was observed in the rodent motor cortex. No detectable labeling was observed in the three sets of control sections; incubated with the non-biotinylated probe, pretreated with RNase, or the absence of probe.

The putative interactions between dopaminergic afferents and cortical target neurons determined from our data are summarized in the diagram in Figure 11.

Figure 9

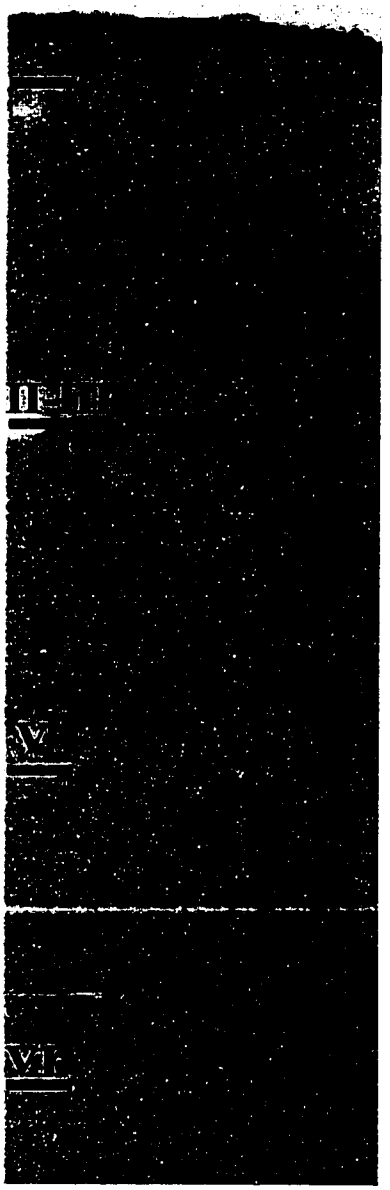


Figure 10

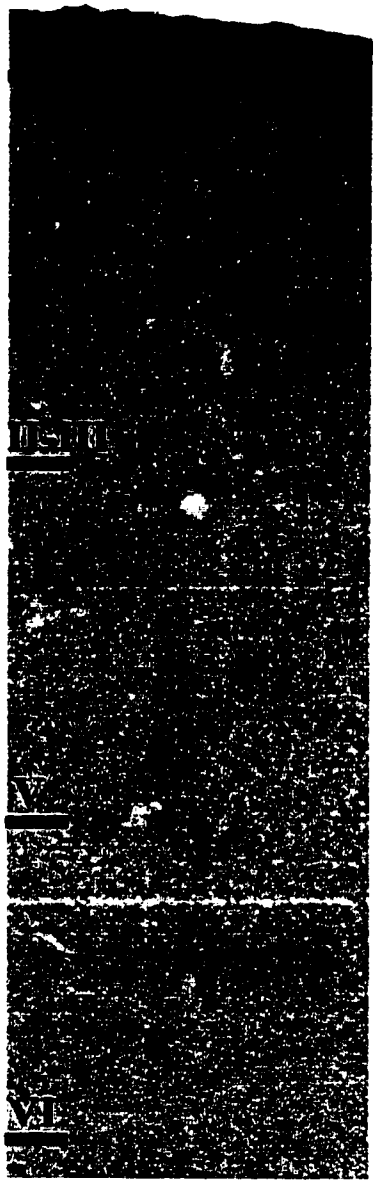


Figure 9. Montage of a section of rodent motor cortex that has been labeled for D1a mRNA. Neurons that contain D1a mRNA are visible throughout layers II-VI.

Figure 10. Montage of a section of rodent motor cortex that has been labeled for D2 mRNA. Neurons that contain D2 mRNA are most dense in the superficial layers II-III, and in some neurons are visible in layer V.

Figure 11

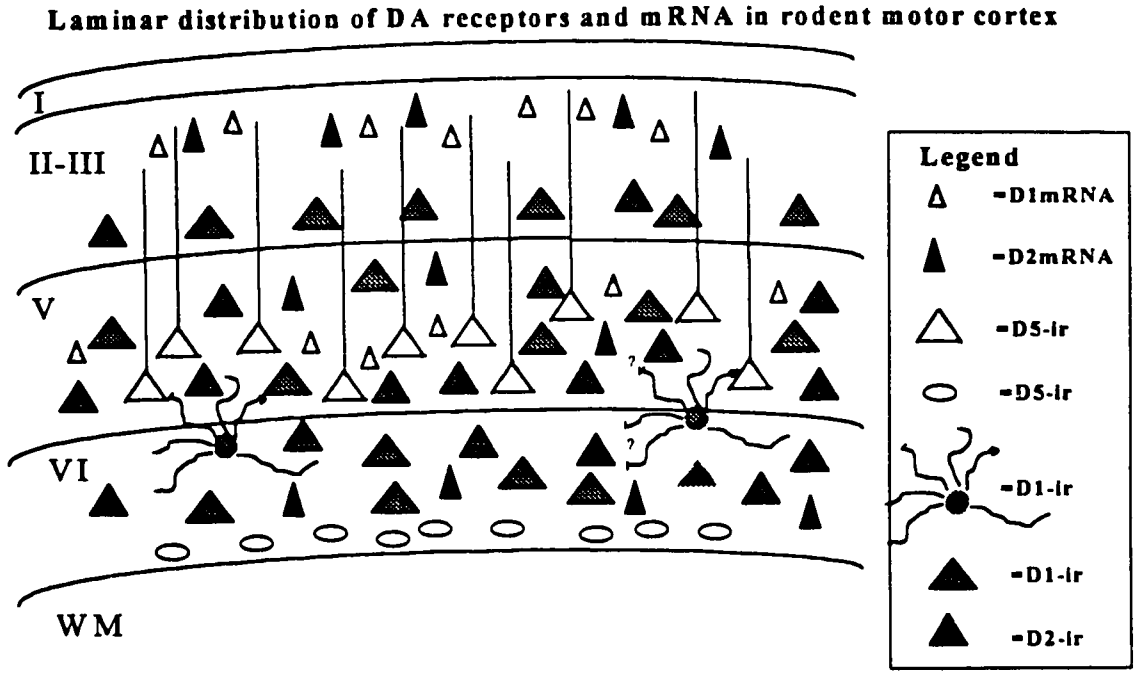


Figure 11. An illustration of the putative interactions between dopaminergic axons and target neurons in rodent motor cortex. Our results indicated that D1a, D2, and D5 receptor protein and mRNA was located within neurons throughout the cortical laminae. D1a receptor protein was also observed within non-pyramidal neurons in layers, V-VI. Additionally, our study revealed that high levels of D1a, D2, and D5 receptor protein were located within numerous large pyramidal neurons in layer V.

FB coupled with receptor immunohistochemistry

A dense band of retrogradely labeled PTNs was evident in all histological sections through the motor cortex after FB injections into the corticospinal tract. The FB label was clearly visible in the somata and proximal dendrites of PTNs. However, FB did not extend into distal dendrites. The distribution of the three receptor subtypes was the same as that described above.

D1a receptors

Numerous D1a-ir neurons were observed in the vicinity of PTNs in layer V of rodent motor cortex. Labeling was observed in the somata and proximal dendrites, including the proximal portion of many apical dendrites, suggesting that most labeled neurons were pyramidal neurons. Many but not all, of the FB labeled PTNs were immunoreactive for D1a receptors, suggesting that a subpopulation of PTNs contains D1a receptors. Examples of FB labeled PTNs immunoreactive for the D1a receptor are shown in Figure 12 A-B.

D2 receptors

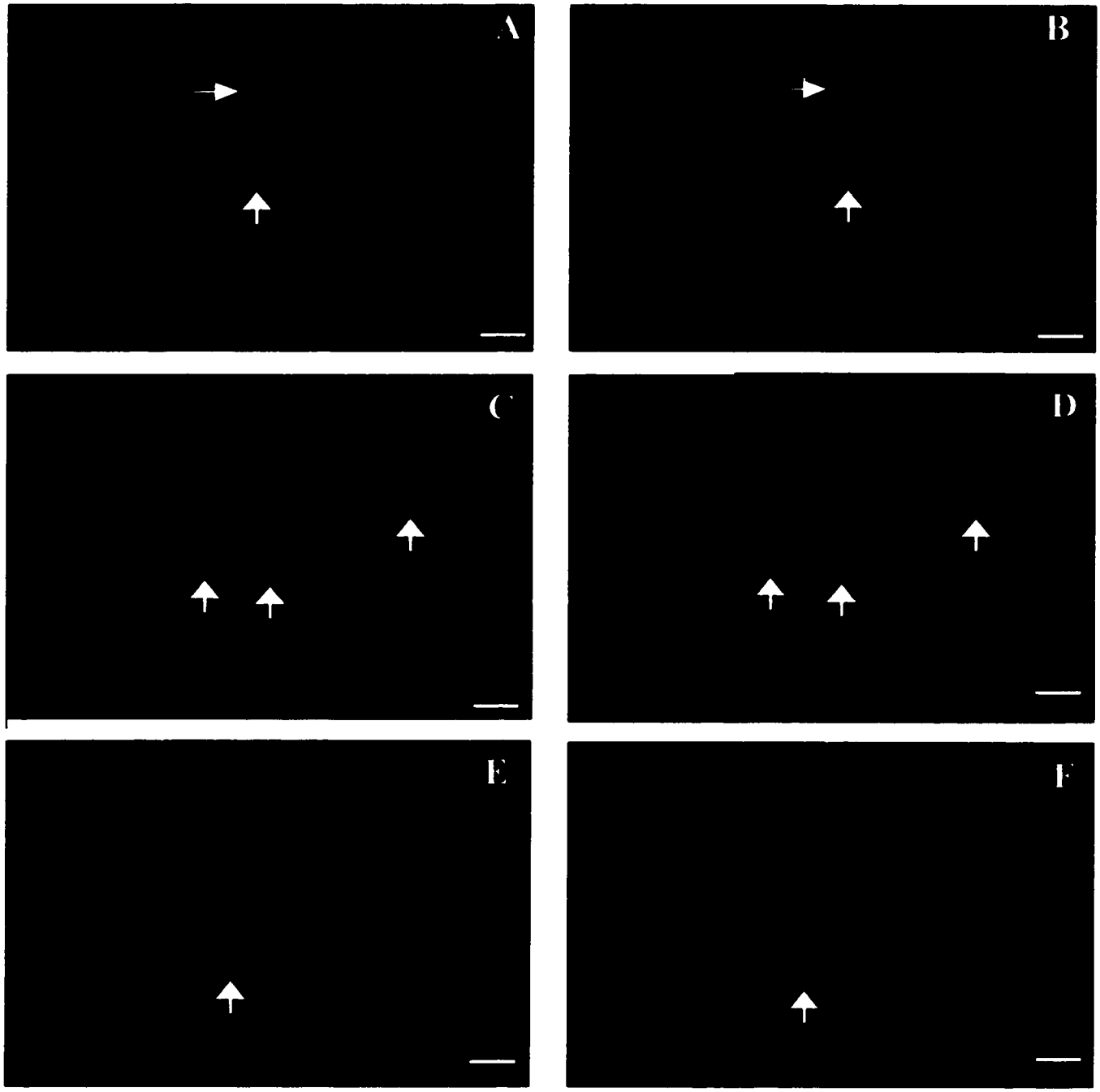
D2-ir neurons were observed primarily within layer V. Here again, labeling revealed the characteristic somal shape and apical dendrites of pyramidal neurons. A large proportion of the FB labeled PTNs were immunoreactive for D2 receptors, indicating that a subpopulation of PTNs contains label for D2 receptors. Examples of FB labeled PTNs immunoreactive for the D2 receptor are shown in Figure 12 C-D.

D5 receptors

Numerous D5-ir neurons were observed in layer V. Labeling was observed in the somata, as well as in extensive portions of dendrites, particularly the apical dendrites. This observation allowed us to identify these neurons as pyramidal cells. Many of the FB labeled PTNs were immunoreactive for D5 receptors, but some PTNs did not label for D5 receptors. Examples of FB labeled PTNs

Figure 12. Photomicrographs of FB labeled PTNs in rodent motor cortex that contain D1a, D2 or D5 receptors. A) Photomicrograph of FB labeled neurons. B) Photomicrograph of same field as in A showing D1a-ir neurons. Arrows in A and B indicate FB labeled PTNs immunoreactive for D1a receptors. C) Photomicrograph of FB labeled neurons. D) Photomicrograph of same field as in C, showing D2-ir neurons. Arrows indicate FB labeled PTNs immunoreactive for D2 receptors. E) Photomicrograph of FB labeled neurons. F) Photomicrograph of same field as in E, showing D5-ir neurons, note the label on the apical dendrites. Arrows in E and F, indicate FB labeled PTNs immunoreactive for D5 receptors (scale bar = 50 μm for A - F).

Figure 12



immunoreactive for the D5 receptor are shown in Figure 12 E-F.

Discussion

These experiments showed that the DA D1a, D2, and D5 receptor subtypes were abundant in the rodent frontal cortex. D1a, D2, and D5 receptor proteins were expressed predominantly by pyramidal neurons spanning layers II-VI, but also by non-pyramidal neurons in layers V-VI. Neurons containing different subtypes exhibited distinct laminar distributions throughout the depth of the cortex. In most neurons, label was detectable only in somata and proximal dendrites. However, in the D5 containing neurons, label extended far into the apical dendrites, and in the D1a containing non-pyramidal neurons, an extensive portion of the dendritic arbor was labeled. These experiments also demonstrated that DARPP-32 was co-localized with D1a receptors in all D1a-ir non-pyramidal neurons and with many D1a, D2, and D5-ir in pyramidal neurons in layers V-VI. The distribution of D1a and D2 mRNA was similar to that seen for the D1 and D2 receptor proteins. A relative paucity of D5 mRNA was observed in all cortical layers of rodent motor cortex.

The results also showed that PTNs possess different DA receptors subtypes. The D1a, D2, and D5 receptors were all expressed by PTNs, although each receptor is not ubiquitously expressed by the entire population of PTNs.

Technical considerations

The two probes used for D1a immunostaining resulted in markedly

different labeling patterns. The construction of the two antibodies for D1a should explain these variations. The rat anti-D1a probe was created against the human D1a receptor sequence, whereas the rabbit anti-D1a probe was created against the rat D1a receptor. Additionally, the rabbit anti-D1a probe was created against three distinct peptide fragments of the rat D1a receptor, while the rat anti-D1a was a fusion protein, created against one 97 amino acid fragment.

Gaspar and colleagues (1995), using retrograde labeling and *in situ* hybridization, were unable to detect D1a and D2 mRNA in PTNs. These differences from our findings may have arisen from their use of radioactive probes to detect mRNA which did not meet their criteria for positive labeling. Alternatively, they may not have successfully labeled all PTNs. It is also possible that our antibodies may have a higher level of sensitivity, allowing us to identify D1a and D2-ir PTNs.

DA receptor distribution in motor cortex

The laminar distribution of dopaminergic axon terminals in the frontal cortex is such that neurons in all cortical layers are potential targets of this input pathway. Indeed, our studies of DA receptor distribution support this notion, in that neurons throughout the cortical layers contain the mRNA and express proteins for at least one of the receptor subtypes. It was not surprising that an increased density of DA receptors was observed in layers V-VI of rodent motor cortex, because DA innervation is most dense to these layers (Descarries et al., 1987; Berger et al., 1991). Likewise, the lower levels of D1a, D2, and D5

receptors in layers II-III reflect the relatively sparse DA innervation of layers II-III (Descarries et al., 1987; Berger et al., 1991). The laminar distribution of neurons that contain D1a and D2 receptor mRNA observed in the present study was similar to that reported in previous *in situ* hybridization studies (Mansour et al., 1992; Gaspar et al., 1995). In addition, the D1a and D5 receptor distribution patterns in rodent motor cortex reported here were similar to that shown by receptor binding studies in rodent neocortex using the D1 selective radioligand SCH23390 (Boyson et al., 1986; Richfield et al., 1989; Mansour et al., 1992; Vincent et al., 1993). A similar distribution was found in primate neocortex, where numerous D1a (Smiley et al., 1994; Bergson et al., 1995b) and D5-ir neurons span layers II-VI (Bergson et al., 1995b). Surprisingly, D5 mRNA levels were very low in the rodent motor cortex in contrast to the high density of D5-ir neurons. Low levels of D5 mRNA in rodent neocortex has been reported by others (Sunahara et al., 1991; Tiberi et al., 1991), suggesting that our finding was not a result of insensitive probes. Alternatively, the low levels of message may be an indication of a low rate of receptor turnover or and increased rna instability.

The likely targets of DA cannot be predicted however, by the laminar locations of somata that contain the mRNA for DA receptors or the expressed protein. We found D5 immunoreactivity in the portions of apical dendrites that originated from somata in both, layers II-III and layers V-VI, but which extended into layer I. Synaptic contacts have been observed in layer I of primate cortex,

between DA terminals and dendritic spines presumed to belong to terminal branches of apical dendrites (Goldman-Rakic et al., 1989). Although immunoreactive label in distal dendrites and spines could not be detected with our methods, ultrastructural studies suggest that they exist (Muly et al., 1998). Thus, neurons in all layers are positioned to receive synapses from the dense DA terminals in layer I, as well as from the layers of their somal origins. It also has been suggested that DA acts in part through non-classical synapses in a paracrine manner (Garris and Wrightman, 1994). Some DA transporters are distant from DA axon terminals (Sesack et al., 1998) allowing DA to activate receptors distant from its release sites. Thus, the laminar location of neurons may not be correlated to the relative potency of DA's effects, and neurons in virtually all layers may be vulnerable to dopaminergic modulation through synaptic and/or paracrine mechanisms.

Pyramidal neurons are the predominant target of mesocortical DA afferents in primates and rodents (Goldman-Rakic et al., 1989; Verney et al., 1990; Smiley and Goldman-Rakic, 1993; Krimer et al., 1997). Accordingly, many of the neurons immunoreactive for D1a, D2, and D5 receptors in our study were identified as pyramidal cells by their somal shape and the presence of a prominent apical dendrite. Many D1a and D5 receptors have been identified in pyramidal neurons of primate frontal cortex also. The abundance of D1a-ir and D5-ir pyramidal neurons throughout the depth of the cortex suggest that DA has a modulatory effect on extensive numbers of pyramidal neuron generated

cortico-cortical and cortico-fugal projections. Because pyramidal neurons use the excitatory neurotransmitter glutamate, DA acting at D1a, D2, or D5 receptor sites has the potential to directly modulate motor cortex excitatory activity.

DA receptors are present in PTNs

Our observation that PTNs possess different subsets of DA receptors show one mechanism through which DA can affect excitatory activity within the cortex and alter corticofugal output. All three receptor subtypes were observed in PTNs. Similarly, Betz cells, a subpopulation of PTNs have also been shown to express D1a, D2, and D5 mRNA's in monkey motor cortex (Huntley et al., 1992). It appears however that DA's effects are not homogeneous for the entire population of PTNs because not all PTNs possess a single type of DA receptor, and in fact some PTNs may possess no DA receptors. Immunostaining for each of the receptor subtypes was evident in only a subpopulation of PTNs. If the receptor proteins were restricted to distal dendrites and spines of some PTNs, then we would not have detected it with our light microscope immunohistochemical techniques, as we were unable to visualize dendritic spines with both the immunohistochemical and FB labels. Previous ultrastructural studies indicate that D1a receptors are located on dendritic spines (Smiley et al., 1994; Bergson et al., 1995b), while D5 receptors are located on dendritic shafts (Bergson et al., 1995b) of pyramidal neurons in monkey prefrontal cortex. Therefore, we cannot rule out the possibility that all PTNs contain at least one receptor subtype. It remains clear however, that multiple

receptor subtypes are found throughout the population and will likely result in varied responses to DA

We did not attempt to determine if multiple receptor subtypes were expressed by individual PTNs. The antibodies for D1a and D2 receptors were both made in rabbits, thereby excluding them from double marker studies. However, unidentified pyramidal neurons previously have been shown to express multiple DA receptor subtypes, in that co-localization of D1a and D5 receptors has been observed within individual neurons of monkey prefrontal cortex (Bergson et al., 1995b).

Numerous layer V pyramidal neurons were immunoreactive for D1a, D2, and D5 receptors but were not labeled with FB, indicating that these unidentified neurons belonged to other classes of cortical neurons. The corticocortical and corticostriatal neurons of layer V that express D1a and D2 receptor mRNA (Gaspar et al., 1995) might have accounted for some of the non-FB labeled neurons. Both of these populations of neurons express two types of DA receptors, but it has not been determined whether individual cortico-cortical or corticostriatal neurons express D1a and D2 receptor mRNA. Therefore, PTNs are not unusual in that as a population, they possess multiple DA receptors. This arrangement indicates a potential for great diversity of DA effects on a single class of neurons. Dopamine D1 and D2 like receptors have opposing actions on the enzyme adenylyl cyclase and activate different signal transduction cascades (Kebabian and Calne, 1979; Seeman and VanTol, 1994). If individual PTNs

possess both types of receptors, then DA's effect on cortical activity may be correlated to the balance of receptors within neurons. Alternatively, physiologically distinct subpopulations of PTNs may be distinguished by the presence of unique DA receptor subtype(s). In this latter instance, the overall effect of DA on motor cortex activity would depend on which neurons are activated by the neurotransmitter.

DARPP-32 co-localization with DA receptors

DARPP-32 co-localizes with D1a, D2, and D5 receptors in distinct neuronal populations. Our finding that many DARPP-ir neurons were not D1a-ir is in accordance with findings in striatal tissue, where only half of the DARPP-ir neurons co-localize D1a receptors (Langley et al., 1997), suggesting that DARPP-32 is co-localized with other receptors. DARPP-32 is phosphorylated by cAMP, therefore it is likely that other neurotransmitters which result in increased cAMP levels may also activate DARPP-32. Furthermore, it appears that DARPP-32 may participate in the signal transduction pathways of D1a, D2, and D5-ir neurons.

DA receptors are present in some non-pyramidal neurons

Our observations that D1a receptors are expressed by non-pyramidal neurons are in accord with ultrastructural findings that DA axons target non-pyramidal, as well as, pyramidal neurons in the neocortex (Goldman-Rakic et al., 1989; Verney et al., 1990; Smiley and Goldman-Rakic, 1993; Sesack et al., 1995; Krimer et al., 1997; Porter, 1995). The inhibitory neurotransmitter GABA is

utilized by most non-pyramidal neurons that form local axonal connections within the neocortex. Both D1 and D2 receptor mRNA has been detected in GABA'ergic interneurons in the rodent neocortex (LeMoine and Gaspar, 1998). Furthermore, a specific population of GABA'ergic interneurons has been identified as targets of DA axons. They compose a subpopulation of neurons that express the calcium binding protein parvalbumin (PV) (Porter, 1995; Sesack et al., 1998). Parvalbumin is co-localized with D1 (Muly et al., 1998) and D4 (Mrzljak et al., 1996) receptors in primate frontal cortex and with D1 and D2 mRNA in rodent neocortex (LeMoine and Gaspar, 1998). Therefore, we expected that D1a receptors would co-localize with PV and assessed this possibility with double label immunostaining techniques. Surprisingly, no double labeled neurons were observed. Further studies are necessary to determine if a population of PV-ir neurons that express D4 receptors, also exist in the rodent motor cortex.

The extensive somal and dendritic labeling of the D1a-ir non-pyramidal neurons that we observed, suggests that DA may have a potent and selective effect on these few cells. Little information is available, but DA suppresses GABA'ergic synaptic transmission in slices of rodent prefrontal cortex (Law-Tho et al., 1994). Interneurons have a significant role in the modulation of intrinsic cortical circuits. Their inhibitory connections in the motor cortex have been implicated in the regulation of task-related neuronal activity (Matsumura et al., 1992) and in the rapid onset of plastic changes in the cortex in response to

different manipulations (Jacobs and Donoghue, 1991). Therefore, DA D1a mediated modulation of interneuron activity may have a profound effect on these inhibitory functions within the cortical circuitry.

Our findings suggest that DA can differentially affect cortical neurons that have unique roles in cortical function. It appears that specific populations of pyramidal and non-pyramidal neurons contain unique receptor subtypes. The morphological relationships that we have observed will help in determining DA's role in modulation of cortical activity and how pathological alterations in cortical DA may contribute to symptomology of relevant disease states.

Our findings also suggest that DA acting at D1a, D2, or D5 receptors on PTNs may have potent effects on skilled movements. Furthermore, a depletion of DA in the motor cortex, as seen in Parkinson's disease, could disrupt normal cortical control of motor behavior through loss of its effects on PTNs.

SPECIFIC AIM 3

The third **specific aim** was to determine how DA affects PTN activity and the receptor subtypes that mediate these effects.

Introduction

PHYSIOLOGICAL EFFECTS OF DA ON PTNS

The physiological effects of DA in modulating cortical activity are uncertain, as both excitatory and inhibitory neuronal responses to DA have been reported (Reader et al., 1979; Bernardi et al., 1982; Bradshaw et al., 1985; Sawaguchi et al., 1986a). Dopamine induced inhibition occurs in neurons across cortical laminae. Inhibitory effects have been demonstrated as DA induced increases in firing threshold coupled with decreases in firing frequency in pyramidal neurons of rat prefrontal cortex (Geijo-Barrientos and Pastore, 1995), and by decreases in task related activity in intact primate frontal cortex (Bernardi et al., 1982; Sawaguchi et al., 1986a). On the other hand, excitation occurs predominantly in neurons of layer V (Sawaguchi et. al., 1986b; Yang and Seamans, 1996). Excitatory effects have been demonstrated *in vivo* as increased spontaneous activity (Sawaguchi et al., 1986a) and *in vitro* as slight DA induced increases in firing frequency following current induced depolarization of cortical pyramidal neurons (Penit-Soria et al., 1987). These varied effects of DA on cortical neuronal activity appear to be somewhat layer dependant, perhaps reflecting the distinct laminar patterns of its associated receptor subtypes. These varied findings, paired with the paucity of studies on DA's effects in the motor

cortex, demonstrate the need for further investigation of how DA modulates motor cortex activity.

Our findings of a widespread distribution of DA receptors in neurons in the motor cortex indicate that DA acts directly through receptor mediated mechanisms on some neurons, especially pyramidal neurons. Only a few studies have focused on the roles of DA receptors in cortical responsiveness to DA. For example, DA reduces the number of spikes elicited by depolarizing current steps in layer V pyramidal neurons of rodent prefrontal cortex. This effect is mediated by D2 receptors (Gulledge and Jaffe, 1998). D2 receptors are also implicated in mediating the excitatory effects of DA on cortical neurons (Bradshaw et al., 1985). A contradictory report suggests that D1, but not D2 agonists elicit decreases in spike latency and increases in firing frequency in layer V pyramidal neurons (Yang and Seamans, 1996). While the results of these reports are varied, they clearly implicate DA receptors in mediation of cortical responses.

Undoubtedly however, other mechanisms also are involved in dopaminergic modulation of cortical activity. Dopamine induced depolarization of rodent prefrontal cortex neurons could not be mimicked or blocked by several common DA agonists or antagonists, respectively (Shi et al., 1997), leaving the authors to suggest a non-specific mechanism involving DA interactions with other neurotransmitters. Indeed, DA has been shown to modulate neuronal responses evoked by glutamate. In slices of human neocortex, DA's influence over glutamate induced responses are dependant upon the activation of either

N-methyl-D-aspartate (NMDA) or quisqualate receptors, which are subtypes of glutamate receptors (Cepeda et al., 1992). Dopamine increases the depolarization and the firing rate, and decreases the latency of NMDA induced excitatory post-synaptic potentials (EPSPs) in layer V pyramidal neurons (Cepeda et. al., 1992). In contrast, DA decreases the depolarization and firing frequency of quisqualate induced EPSPs (Cepeda et al., 1992). In these studies, the postsynaptic response to DA is dependant on the glutamate receptor subtype involved. Whether these DA-glutamate interactions occur through DA receptor activation is uncertain. Support of such an assumption arises from a report that DA reduces NMDA-induced EPSPs through D1 receptor mediated mechanisms in pyramidal neurons in layer V of rodent prefrontal cortex (Law-Tho et al., 1994). Our studies explored the involvement of DA receptors in activation of PTN spontaneous activity (SA), and the interaction between DA and glutamate-induced excitation of PTNs.

Three distinct DA receptor subtypes, D1a, D2 and D5, have been identified in cortical layer V. Our studies have shown that all three receptors are expressed in PTNs suggesting that DA directly activates PTNs through any one of these receptors. Therefore, it is likely that dopamine's influence over PTN activity is involved in cortical control of motor behavior, as PTNs provide a direct link between the motor cortex and spinal cord motor neurons (Phillips and Porter, 1977). The present study was designed to elucidate DA's influences on PTNs. Electrophysiological techniques were used to determine the effects of DA

on the spontaneous activity of PTNs and the receptors that mediate these effects. In addition DA's modulation of glutamate induced excitation of PTNs was assessed.

Methods

Surgical Procedures

Ten adult Sprague Dawley rats were anesthetized with Xylazine (2-4 mg/kg; im) and Ketamine HCl (60 mg/kg; im) for the surgical procedures. An injection of Dexamethasone (2 mg/kg; im) was administered one hour prior to surgery to prevent cortical swelling. The animals received supplemental injections of anesthetics (30 mg/kg of Ketamine HCl; im and 1.0 mg/kg Xylazine; im) at one hour intervals during the experimental procedure to maintain a steady state of anesthesia. They were checked periodically for the absence of withdrawal reflexes. The animals were placed in a stereotaxic apparatus. Body temperature was maintained at 37°C with a thermoregulating heating pad. An injection of a local anesthetic, xylocaine (1 ml; 20 mg/ml), was made subcutaneously in the area of the incision sites. A midline incision was made. A craniotomy and durotomy were done unilaterally, to expose the right motor cortex. Warm, sterile mineral oil was maintained on the exposed cortical surface. A laminectomy of the cervical spinal cord was made at the C1-C2 level, to expose the corticospinal tract (CST).

Electrophysiology

A tungsten-in-glass microelectrode (with an exposed tip of 10-15 μm) was mounted onto the carrier of a motorized hydraulic microdrive and lowered into the motor cortex at the site of the forepaw representation (Hall and Lindholm, 1974). The electrode was lowered to a depth of approximately 1200 μm, where most of the PTNs are located. Intracortical microstimulation (constant current, 200 μsec pulses, 200 Hz, 90 msec train, at 1/sec) was delivered through the electrode and current intensity was slowly increased until forepaw movement was elicited contralateral to the stimulus. The current intensity (determined as voltage drop across a 1 KΩ resistor) did not exceed 60 μA at which cortical damage may occur. This step was repeated until a low threshold site for forepaw movement was identified. This site was marked on a drawing of the cortical surface vasculature. The electrode was removed and replaced with a five barrel pipette (tip diameter 12-20 μm). The central barrel contained a carbon fiber (extending < 5 μm from tip) for recording neuronal activity. For the first set of experiments, the four surrounding barrels were filled with either NaCl (2 M) for balancing current, DA (0.1 M), the D1 selective receptor antagonist SCH23390 (SCH, 0.1 M), or the D2 selective receptor antagonist eticlopride (Etic, 0.1 M). For the second set of experiments, the barrels were filled with NaCl (1 M), DA (0.1 M), or glutamic acid (Glu, 0.1 M). The drug filled barrels were connected through a silver wire to an Iontophoresis Module (World Precision Instruments), which was used to monitor electrode resistance and to pass current for iontophoresis of the drugs. A retaining current (10-20 nA) was passed through

each of the drug filled pipettes to prevent leakage during the recordings. The electrode was inserted with the aid of a surgical microscope into layer V of the motor cortex (1000-1300 μm below the pial surface). To accurately determine the depth of the electrode, extracellular neuronal activity was recorded as the electrode was lowered. Neuronal activity was amplified (X 1,000) displayed on an oscilloscope and audiomonitor. A slight change in electrical noise, which occurred as the electrode encountered the mineral oil/cortical surface interface, helped to determine the surface location.

A tungsten-in-glass microelectrode, placed in a carrier attached to the stereotaxic apparatus, was inserted into the left CST (200-400 μm in depth) at the C1 level of the spinal cord (Paxinos and Watson, 1986). The stimulus pulses were passed through the electrode while recording antidromic responses from the electrode placed in the motor cortex. When an action potential was detected in response to the stimulus, tests were applied to ensure that it was antidromic, elicited by stimulating the axons of the PTNs. Our criteria for determining antidromic stimulation was that the neuron exhibited high frequency following in response to the stimulus and the response latency did not decrease with increased current intensity of at least two times threshold. The response latency was expected to be 1.5 to 4.0 msec (axonal conduction velocity/distance between stimulating and recording electrodes). Figure 13 is a diagram of the experimental paradigm.

Figure 13

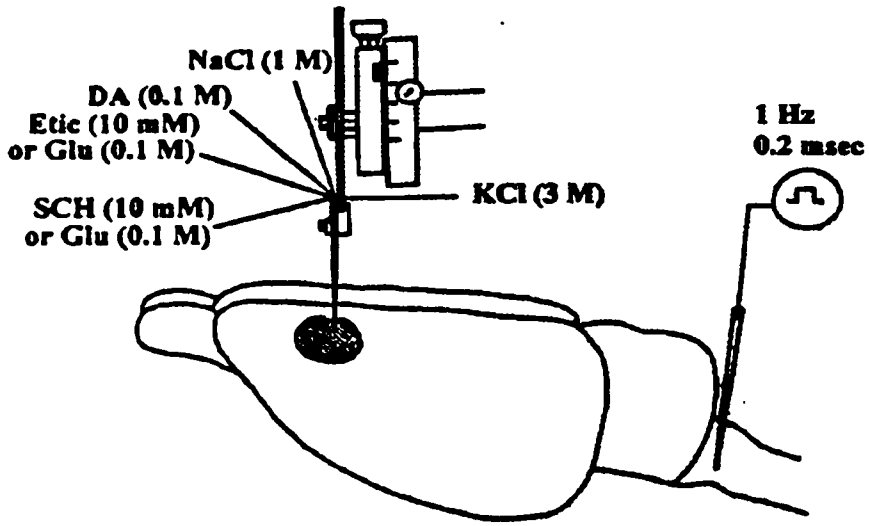


Figure 13. Diagram showing the location of the five barrel pipette in the forepaw representation of the motor cortex, and the substances loaded into the barrels. A stimulating electrode is shown in the opposite corticospinal tract.

Data acquisition

Extracellular recordings were made of the neuronal activity of identified PTNs. Signals were amplified (X 1,000), filtered (low pass = 10,000 and high pass = 300 Hz), displayed on an oscilloscope and audiomonitor. Single or multiple unit activity was partially isolated through a window discriminator (amplitude only). Each action potential that passed through the discriminator triggered a pulse that was recorded to a data acquisition and analysis system (Datawaves). Spontaneous activity of identified PTN's was recorded for intervals of 150 seconds while different drugs were applied over repeated trials. Two separate sets of recordings were made; one using DA and D1 or D2 antagonists,

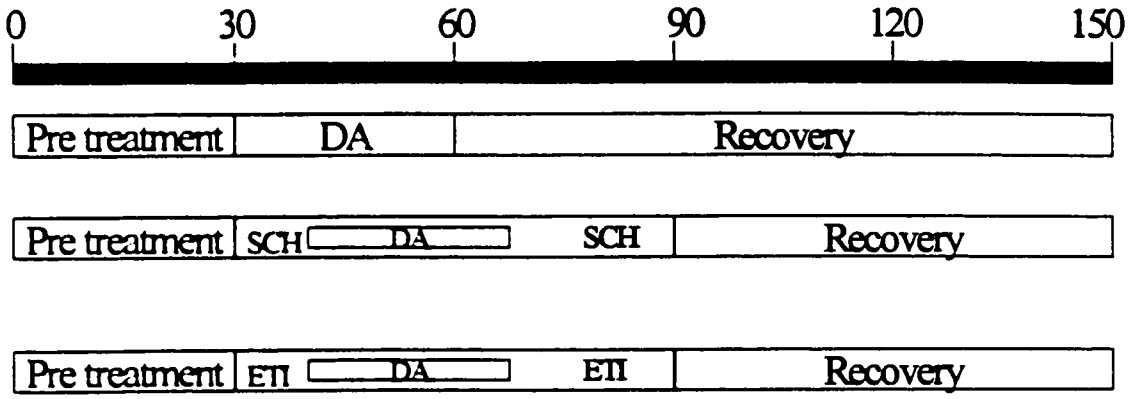
the other using DA and Glu.

Drug application

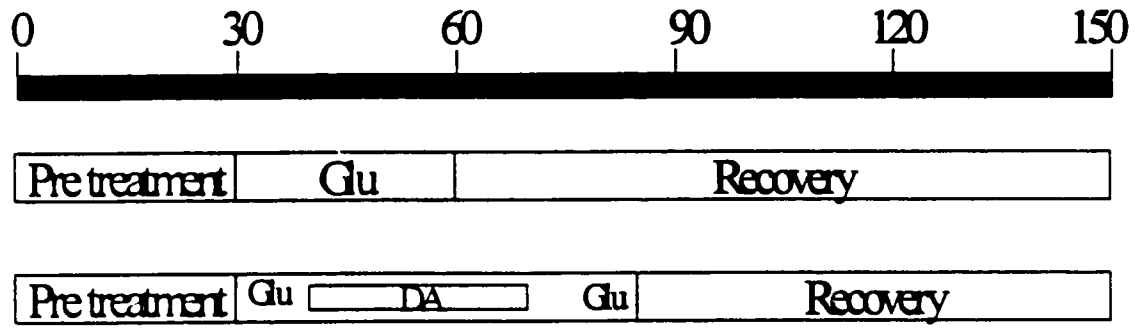
For each PTN in the first set of recordings, drugs were applied in successive trials in three different schedules; 1) baseline recording from 0-30 seconds, DA (+170 nA) from 30-60 seconds, baseline from 60-150 seconds; 2) baseline recording from 0-30 seconds, SCH23390 (+90 nA) from 30-90 seconds, DA from 40-70 seconds, baseline from 90-150 seconds; 3) baseline from 0-30 seconds, eticlopride (+90 nA) from 30-90 seconds, DA from 40-70 seconds, baseline from 90-150 seconds. Each of the three trials was repeated multiple times for each PTN. Trial sequences were randomly ordered. An interval of at least three minutes between trials was included to ensure that SA returned to baseline.

For each PTN in the second set of recordings, drugs were also applied in three different successive trials; 1) baseline recording from 0-30 seconds, DA from 30-60 seconds, baseline from 60-150 seconds; 2) baseline recording from 0-30 seconds, Glu from 30-60 seconds, baseline from 60-150 seconds; 3) baseline recording from 0-30 seconds, Glu from 30-80 seconds, DA from 40-70 seconds, baseline from 80-150 seconds. Each of the three trials was repeated multiple times for each PTN. Trial sequences were randomly ordered. An interval of at least three minutes between trials was included to ensure that SA returned to baseline. Neurons whose firing rates did not respond to glutamate were excluded from further testing.

Table 1.



Treatment schedule for DA and antagonists



Treatment schedule for DA and glutamate

Table 1. A diagram that shows the timing of drug application for each trial in the two sets of recording experiments. The filled bar at the top of each trial shows the time scale (0 - 150 seconds). The open bars show the time over which drugs were applied for each PTN.

Data Analysis

At the end of the recording sessions, the animals were euthanized by intracardial perfusion with saline, followed by 4% paraformaldehyde in phosphate buffer. Data analysis was performed off line. Single unit activity was discriminated using the Datawaves analysis system which matches parameters

of wave form shape to identify single units. The firing rate (spikes/second) of each PTN during the baseline, drug application, and recovery periods of each recording interval was calculated to determine the effects of drugs on SA. The occurance of action potentials over the 150 second recording interval were displayed as rate meters SA.

Controls

A baseline recording with no drug treatment was made for each identified PTN to examine normal fluctuations of spontaneous activity. SCH23390 and eticlopride were applied alone to determine if they had any effect on spontaneous activity. Pretreatment baseline intervals served as internal controls for each recording.

Statistical Analysis

Data was summed over multiple trials for each PTN. The changes in SA during each treatment interval were compared for individual PTNs. The summed SA of all PTNs during a given treatment interval, was used in our statistical analysis. The Student's T-test was used to determine the statistical differences between the mean SA during baseline intervals, and either the treatment, or recovery periods for a given treatment.

Results

DA application

Successful trials from 31 spontaneously active PTNs from ten adult

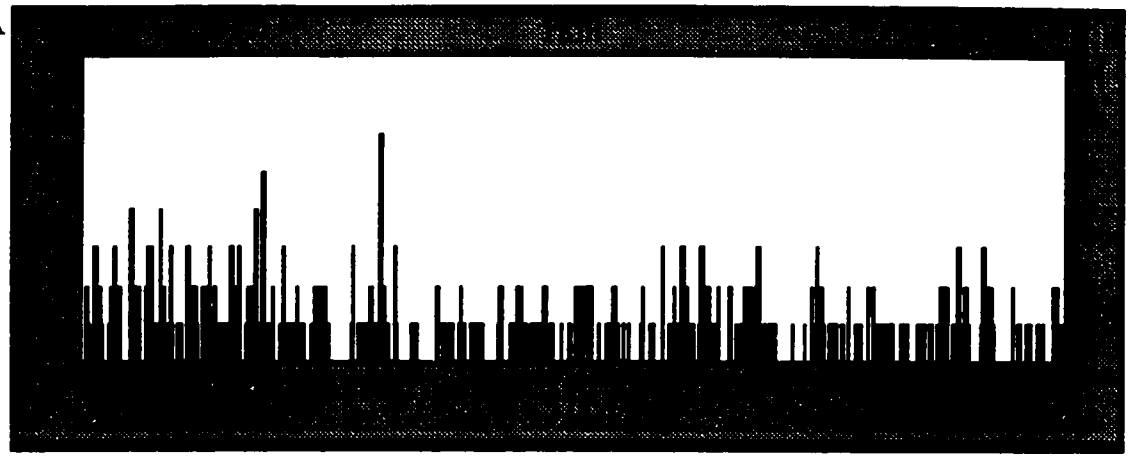
Sprague Dawley rats were completed for the recordings. The response characteristics to CST stimulation of all these neurons met our criteria for antidromically induced action potentials of PTNs. The response latency to CST stimulation of the PTNs was between 1.6 and 3.7 msec (mean = 2.7 msec; SD = 0.7). The mean baseline spontaneous activity of all PTNs tested was 5.4 s/s (SD = 2.8).

Responses to DA application alone over repeated recordings in both the antagonist and the glutamate trials were similar. Dopamine application reduced the mean baseline SA of all PTNs from 5.8 s/s to 4.4 s/s (n = 31 PTNs, 4 trials/neuron; Student's T-test, $p < .001$). This change represents a decrease to 75% of baseline SA. The mean firing rate recovered somewhat in the 30 second period succeeding DA treatment (from 4.4 s/s to 5.0 s/s), but did not fully return to baseline levels until 60 - 90 seconds after treatment. In a few isolated cases, the SA of identified PTNs did not return to baseline levels after drug treatment. These PTNs were excluded from the study. In all other PTNs however, SA returned to baseline levels within one to four minutes after cessation of the drug application. Repeated recordings were not initiated until SA had fully recovered to baseline levels. The Rate meter from a single 150 second recording interval of SA from one PTN is shown in Figure 14 A. A decrease in firing rate during the DA interval is evident. Some recovery occurs during the 30 seconds following drug application and continues beyond this time. The chart in Figure 14 B shows the mean firing rate over four trials for the same neuron, during the pretreatment,

drug application and recovery periods.

Figure 14

A



B

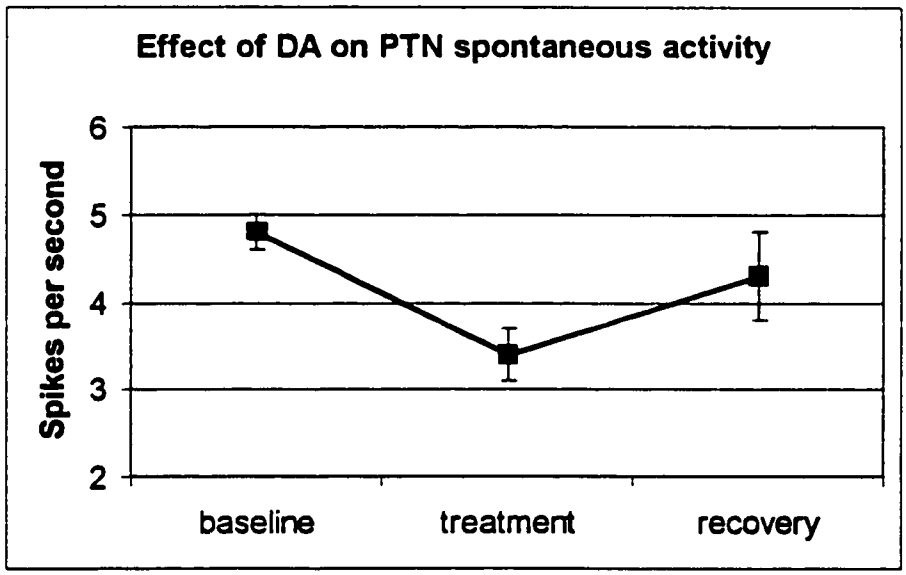


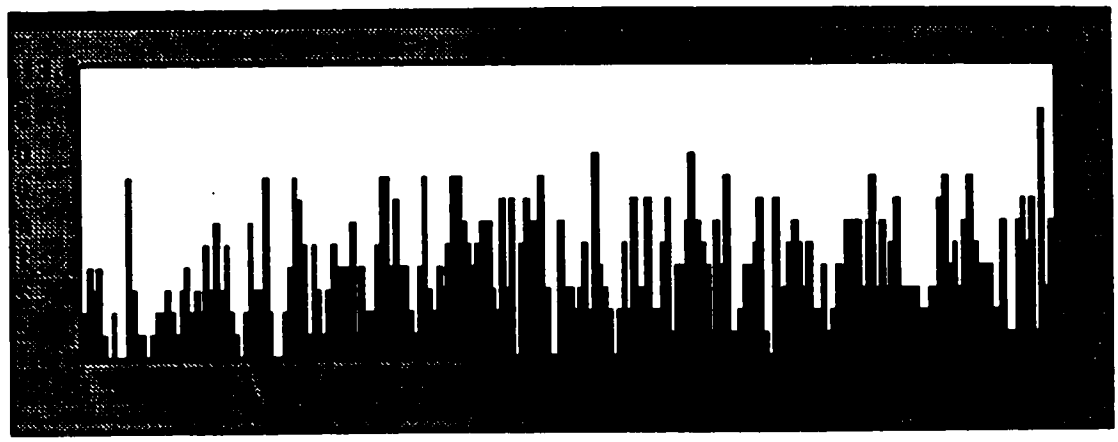
Figure 14. A) Rate meter in real time, showing the SA of one PTN over a 150 sec recording interval (120 sec shown). Baseline activity was recorded for 30 seconds then DA was applied between 30 and 60 seconds (arrows). The firing rate was reduced during this time period. Spontaneous activity did not return to baseline during the 30 second recovery period. B) Chart showing the SA (mean spikes per second) for a 30 second interval of pretreatment, drug application, and recovery, across four trials of pretreatment, drug application, and recovery, of the same PTN as in A (bin width = 400 msec).

DA and antagonist application

Nineteen of the 31 PTNs were used in the DA and antagonists trials. Co-application of SCH23390 with DA blocked the DA induced inhibition in that SA was unaltered from baseline (98% of baseline SA) during the drug application (n = 19 PTNs, 3 trials/neuron; $p > 0.1$). The SA during the post treatment interval also remained unaltered from baseline (106% of baseline SA: n = 19 PTNs, 3 trials/neuron; $p > 0.1$). The Rate meter from one 150 second recording interval of SA from one PTN is shown is Figure 15 A. No decrease in firing rate during DA and SCH23390 co-application is evident. The chart in figure 16 shows the firing rate of this same neuron averaged over three trials, during the pretreatment, drug application, and recovery intervals. Co-application of eticlopride with DA blocked the DA induced inhibition in that SA was unaltered from baseline (102% of baseline) during the drug application (n = 19 PTNs, 3 trials/neuron; $p > 0.1$). The SA during the post treatment interval remained unaltered from baseline (98% of baseline) ($p > 0.1$). The Rate meter from one 150 second recording interval of SA from one PTN is shown is Figure 15 B. No decrease in firing rate during DA and eticlopride co-application is evident. The chart in Figure 16 shows the firing rate of this same neuron averaged over three trials, during the pretreatment, drug application, and recovery intervals. Application of SCH23390 or eticlopride independent of DA did not change SA rates of PTNs.The SA of all neurons during the DA and antagonist applications are summarized in Figure 17.

Figure 15

A



B

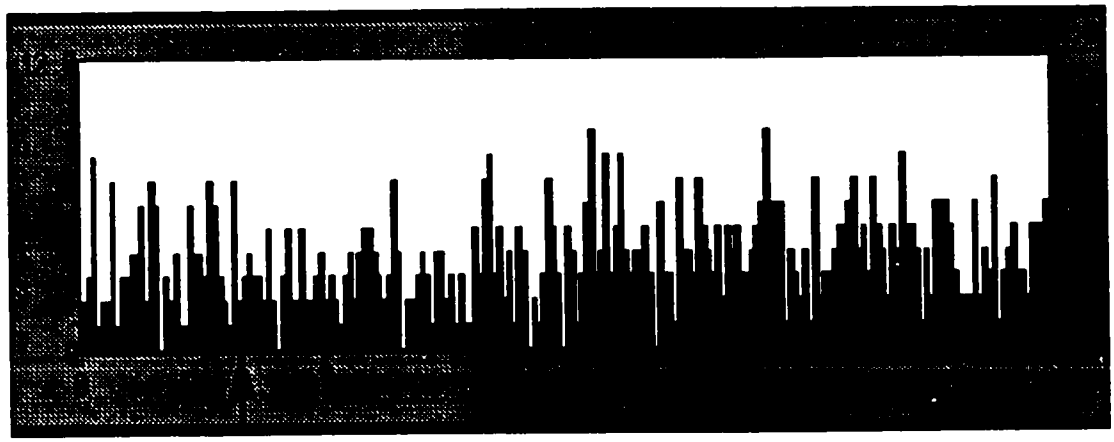


Figure 15. A) Rate meter in real time showing the SA of one PTN during a 150 second recording interval (120 seconds shown). Large arrows indicate SCH23390 application, small arrows indicate DA application. The SA remains near baseline during the co-application of DA and SCH23390. B) Rate meter in real time showing the SA of one PTN during a 150 second recording interval. Large arrows indicate eticlopride application, small arrows indicate DA application. The SA remains near baseline during the co-application of DA and eticlopride (bin width for A and B is 400 msec).

Figure 16

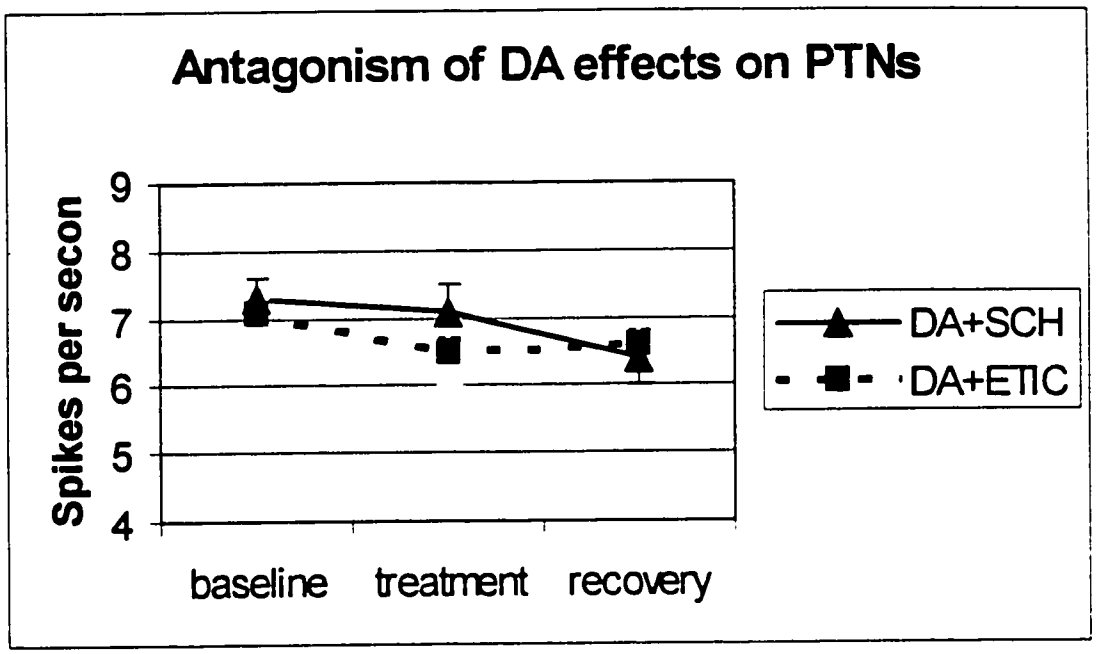


Figure 16. Chart shows the mean SA (spikes per second) for three trials, over the 30 second interval indicated for both antagonists and DA combinations for the same neuron as in figure 15 A-B.

Figure 17.

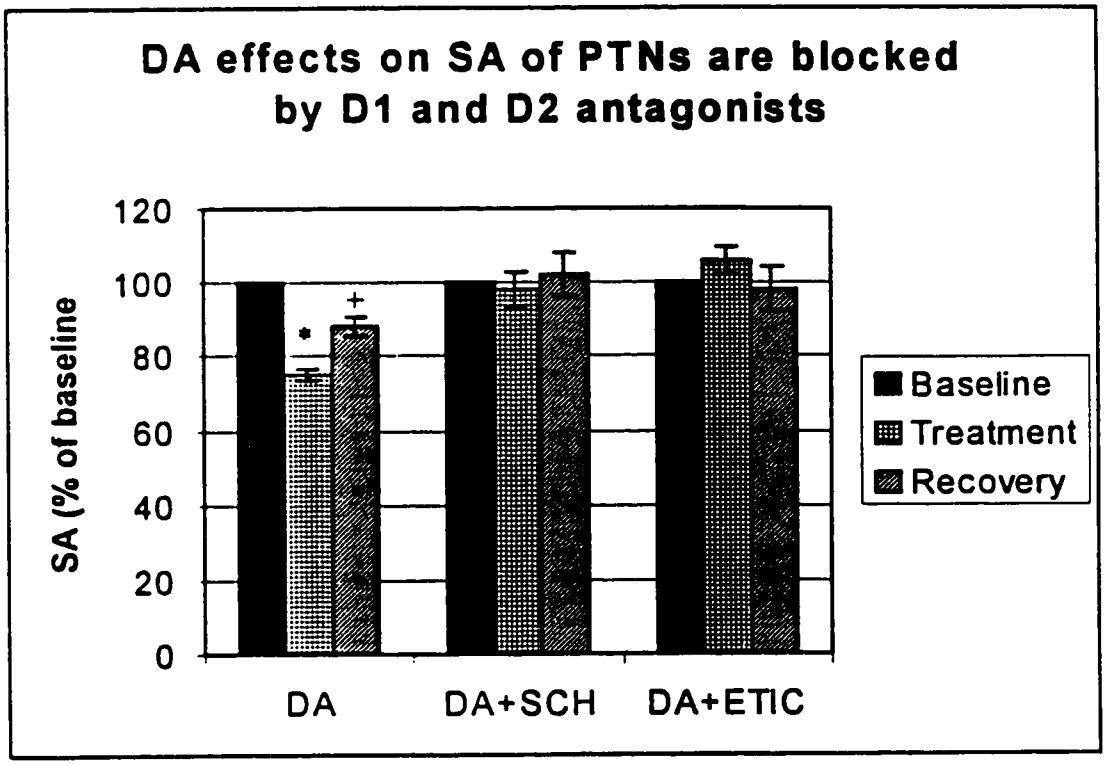


Figure 17. Summary of the effects of DA and antagonists on all PTNs. The mean SA rates (expressed as percent of baseline) are shown for baseline (30 sec), treatment (30 sec), and recovery (30 sec) intervals for trials of DA only, DA + SCH, and DA + Etic. DA reduced the SA of PTNs (n = 31 PTNs, 4 trials/neuron) to 75% of baseline. SCH blocked DA's inhibitory effects (n = 19 PTNs, 3 trials/neuron; 98% of baseline). Eticlopride also blocked DA's inhibitory effects (n = 19 PTNs, 3 trials/neuron; 102% of baseline). * indicates a significant difference from baseline (student's T-test, $p < .001$). + indicates a significant difference from treatment (student's T-test, $p < .001$).

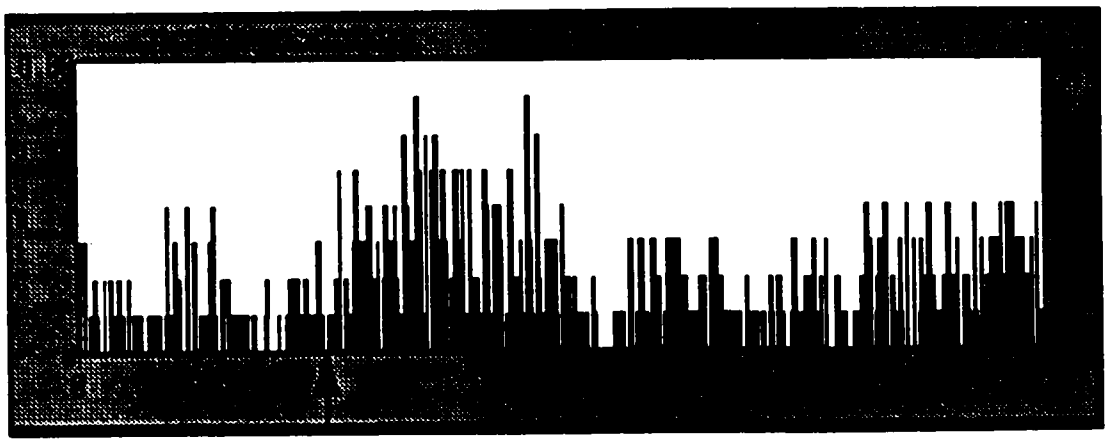
DA and Glu application

The remaining 12 PTNs were used in the DA and glutamate trials. Local application of Glu significantly increased the overall firing rate of PTNs from 4.5 s/s to 6.4 s/s (143% of baseline; n =12 PTNs 3 trials/neuron; Student's T-test, $p < .001$). The Rate meter from one 150 second recording interval of SA from one

PTN is shown is Figure 18 A. An increase in firing rate during Glu application is evident. The chart in Figure 18 B shows the firing rate of this same neuron during the pretreatment, drug application, and recovery intervals. When DA was co-applied with Glu, the Glu induced excitation did not occur in that no change from baseline SA was observed (96% of baseline; $p > .05$). Dopamine reduced the Glu enhanced firing rate from 6.4 s/s to 4.3 s/s, a reduction to 67% of peak Glu induced excitation ($n = 12$ PTNs, 3 trials/neuron; student's T-test, $p < .001$). The baseline SA remained unchanged after the cessation of DA application, even in the 10 seconds of continued application of glutamate (Glu2; 102% of baseline) and for the succeeding 30 second recovery interval (100% of baseline). The Rate meter from one 150 second recording trial of DA and Glu from one PTN is shown is Figure 19 A. An increase in firing rate during the initial Glu application, and a return to baseline during DA application are evident. The chart in Figure 19 B shows the firing rate of this same neuron during the pretreatment, drug application, and recovery intervals. The SA of all neurons during the DA and Glu trials are summarized in figure 20.

Figure 18

A



B

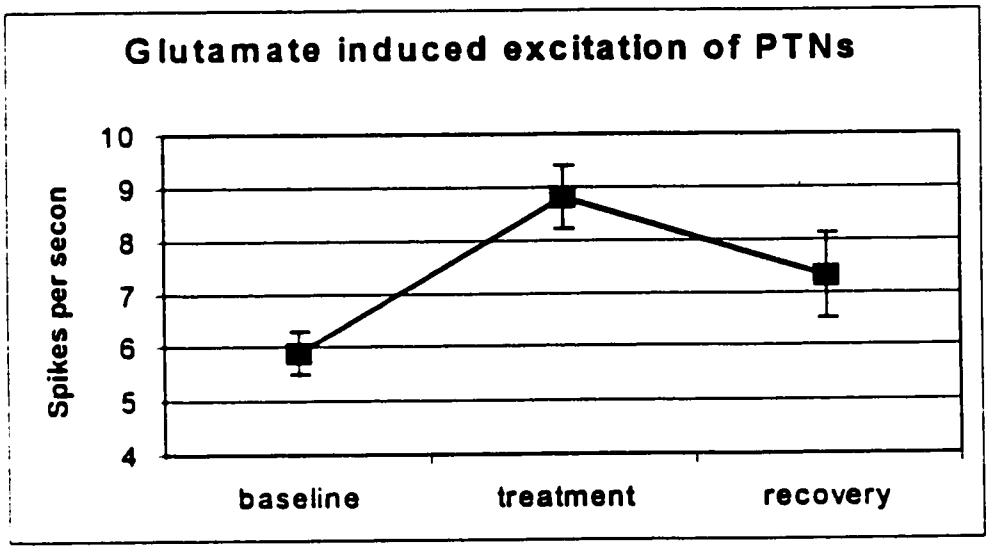
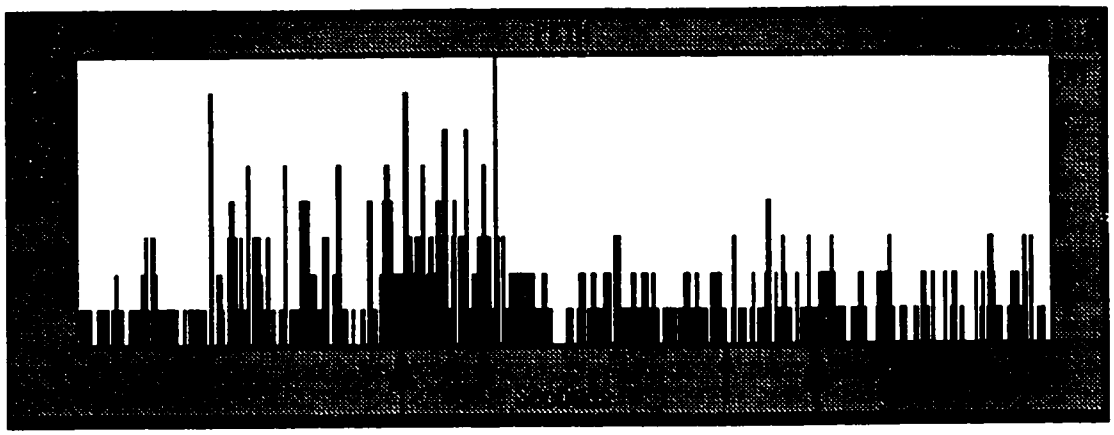


Figure 18. A) Rate meter in real time, shows the SA of a PTN during one trial. Arrows indicate glutamate application (30-60 seconds). Glutamate application caused a marked increase in firing rate. B) Chart shows the mean SA (spikes per second) in each 30 second interval for the same PTN as in A, across three trials.

Figure 19

A



B

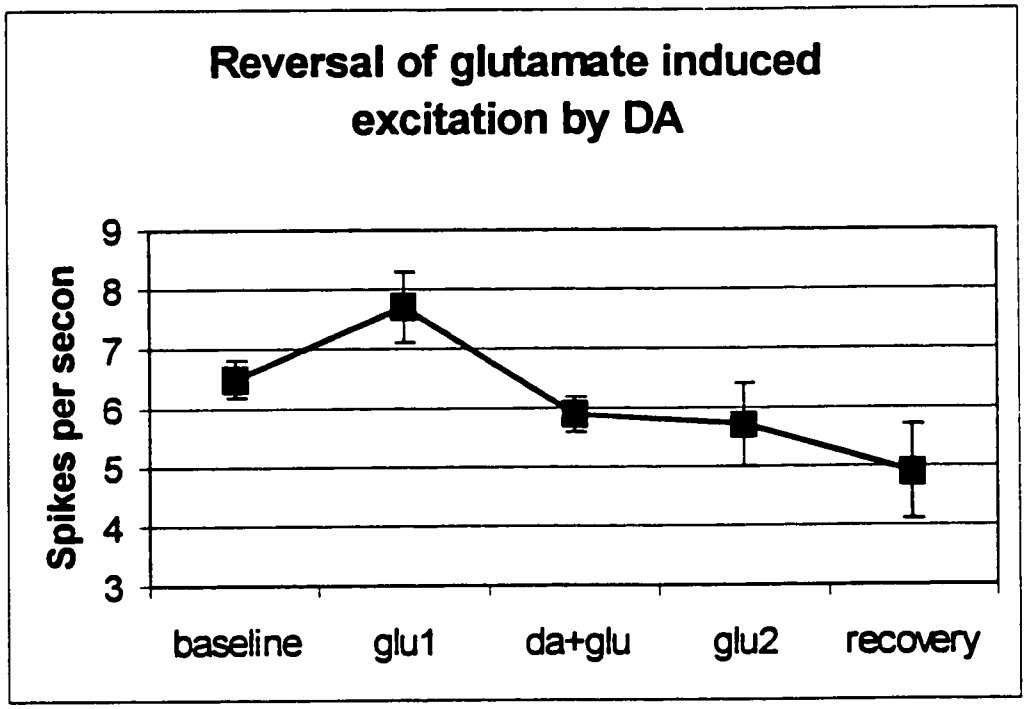


Figure 19. A) Rate meter in real time, shows the SA of a PTN during one trial. Large arrows indicate glutamate application (40-90 secs), smaller arrows indicate DA application (50-80 secs). The glutamate enhancement of SA is visible at the onset of glutamate application (Glu1) but returned to baseline during the application of DA (bin width = 400 msec) B) Chart shows the mean SA (in spikes per second) in each interval indicated for the same PTN as in A, over three trials.

Figure 20

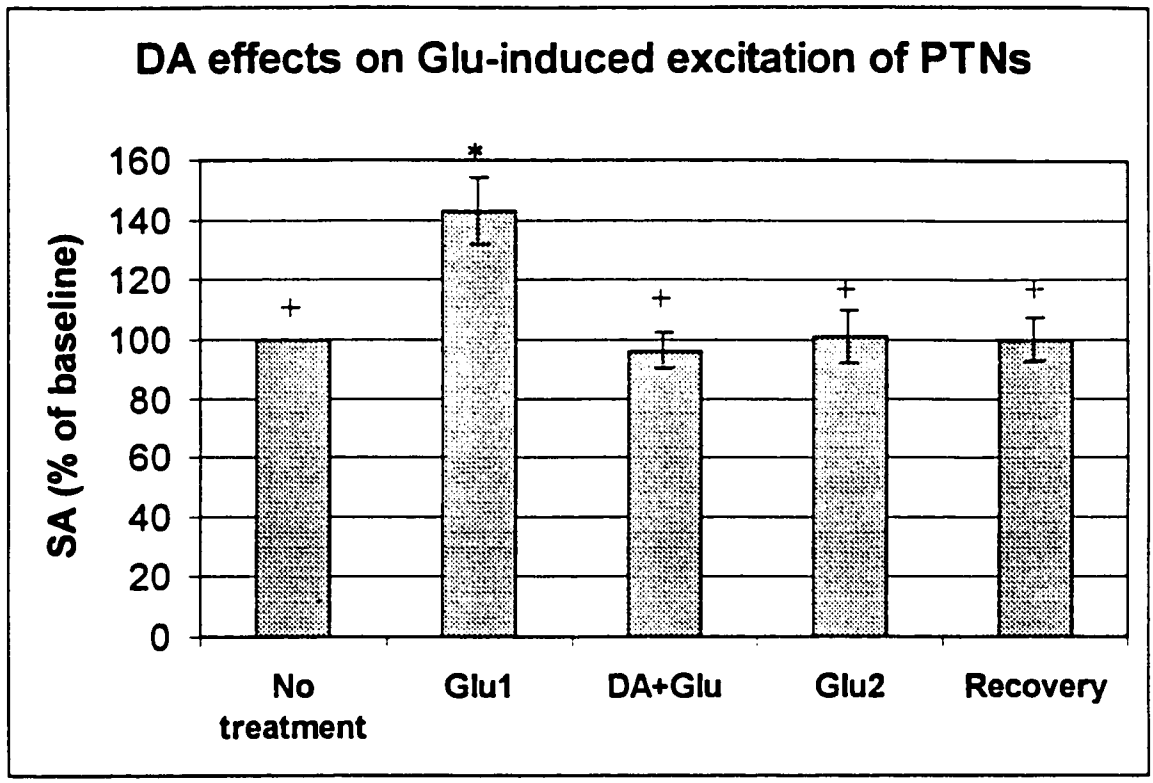


Figure 20. Summary of the effects of DA and glutamate on all PTNs. Data are shown for recordings in which glutamate and DA were co-applied. The glutamate treatment (Glu1) increased the firing to 143% of baseline (n = 12 PTNs, 3 trials/neuron). The co-application of DA with glutamate reversed this increase to 96% of baseline. The firing rate remained unchanged from baseline SA during Glu2 (no DA) during the recovery interval (n = 12 PTNs, 3 trials/neuron).* indicates a significant difference from baseline (student's T-test, $p < .001$). + indicates a significant difference from Glu1 (student's T-test, $p < .001$).

Discussion

Dopamine decreases the SA of identified PTNs in the rodent motor cortex. This effect is non-selectively blocked by both the D1 selective antagonist SCH23390 and the D2 selective antagonist eticlopride. Dopamine also reverses

the glutamate induced increase in SA of identified PTNs.

Technical Considerations

The SA varied across the samples of PTNs used in these recordings. This variability may have been a result of slight differences in anesthetic levels in different animals or in the same animal at different times. Alternatively, inherent properties of individual PTNs may have accounted for the variables. For each PTN, we recorded SA in the absence of drugs to confirm that drug effects on SA were not due to normal variance in SA. In addition, the inclusion of a 30 second pretreatment interval in each recording served as an internal control for variations in SA caused by extèrnal factors, such as anesthesia.

The exact amount of DA expressed by the ejection current was dependant on several factors, including DA concentration, tip diameter, and current intensity. Consistency of all three variables was maintained as accurately as possible. Dopamine concentrations ranging from 0.05-0.8 M and ejection currents ranging from 50-200 nA were used for similar paradigms in previous studies (Reader et al., 1979; Bernardi et al., 1982; Bradshaw et al., 1985; Sawaguchi et al., 1986a,b). We performed a dose-response test during the initial experiment and determined the minimal effective concentration to be 0.1 M DA, with a 170 nA ejection current. Initially, DA was applied for a 10-20 seconds interval, but this duration caused little or no change in SA. Similarly, prior studies in rodent neocortex indicate that the onset of reduction in the firing rate can be delayed from 15 or more seconds from the beginning of DA application (Reader

et al., 1979; Bassant et al., 1990), perhaps because of the delay in reversing the effects of the retaining current. Therefore, we increased the duration of DA application to 30 second intervals which proved to be more effective.

During the recovery interval, SA often returned partially to baseline levels, but full recovery could be delayed more than one minute after cessation of DA application. Other studies, in which DA was iontophoresed, show that its inhibitory effects lasted from one to three minutes beyond the cessation of ejection current (Reader et al., 1979; Bassant et al., 1990). This delay may have been caused by DA's prolonged presence in the extracellular space and would also account for the lack of an increase in firing rate during the Glu2 intervals of the glutamate trials. Dopamine transporters (DAT) are sometimes distant from release sites of dopaminergic axon terminals (Sesack, 1998), a mechanism that delays DA uptake and allows it to activate receptors for a prolonged period of time. In accordance with this notion, cyclic voltammetry studies have shown that clearance of extracellular DA is much slower in the rat frontal cortex than in the striatum (Garris et al., 1993; Garris and Wrightman, 1994). To ensure that DA was cleared from the recording site and that SA recovered to baseline levels, we allowed three to five minutes between each trial.

Dopamine inhibits the SA of PTNs

Previous studies report both excitatory and inhibitory effects of DA on cortical neurons. The present study shows that DA has an inhibitory effect on PTNs. This finding suggests that the outcome of DA application may depend on

the neuronal population upon which it is acting. Our findings that both D1 and D2 receptor antagonists block DA induced inhibition of PTN SA, suggests that some PTNs may possess both receptors. DA induced inhibition of activity on five PTNs was more effectively blocked by SCH23390 than eticlopride, and on four other PTNs was more effectively blocked by eticlopride than SCH23390. This observation suggests not only that our antagonist concentrations were selective, but also that individual PTNs possess only D1 like receptors, only D2 like receptors, or both D1 and D2 like receptors. The different responses elicited by DA from layer V pyramidal neurons (Yang and Seamans, 1996; Gulledge and Jaffe, 1998) and the variable responsiveness of neurons to D1 or D2 receptors may reflect such variations in the presence of distinct receptor subtypes even within a single population of neurons.

Synergistic actions between D1 and D2 receptors in the cortex have been reported previously. Both D1 (SKF38393) and D2 (M RU24926 and M LY171555) receptor agonists inhibit the electrically-evoked release of GABA in rat prefrontal cortex (Retaux et al., 1991). The D1 agonist induced inhibition is fully blocked by both D1 and D2 antagonists (Retaux et al., 1991). Likewise the D2 agonist induced inhibition is fully blocked by both D1 and D2 antagonists (Retaux et al., 1991). These findings indicate either non-selectivity of both agonists and antagonists, or more likely that these DA induced effects are mediated by a synergism of D1 and D2 receptors. A similar receptor synergy may be involved in the dopaminergic modulation of PTNs.

Dopamine and glutamate interactions

The inhibitory effects of DA on cortical pyramidal neurons is thought to be caused in part by the effects of DA on GABAergic interneurons that in turn, impinge on pyramidal neurons (Penit-Soria et al., 1987). We have demonstrated that some interneurons contain DA receptors, which would allow for such a mechanism to function in the motor cortex. In addition, DA receptors have been identified in several populations of pyramidal neurons (Gaspar et al., 1995; present study) indicating that direct DA receptor mediated effects may also function in inhibition of PTNs. Dopamine may also alter neuronal activity by modulation of the excitatory input onto cortical pyramidal neurons. The notion that dopaminergic and glutamatergic axon terminals might function as interactive units, presynaptic to pyramidal neurons, arose from the discovery of the synaptic triads discussed earlier (Goldman-Rakic et al., 1989). The reversal of glutamate induced excitation by DA seen in the present study suggests presynaptic modulation of glutamate induced responses. An *in vitro* study of DA effects on layer V pyramidal neurons in rodent prefrontal cortex, showed that both AMPA and NMDA induced glutamatergic excitatory post synaptic responses were reduced by DA through a D1 mediated mechanism (Law-Tho et al., 1994). Thus our studies support the notion that all three of these mechanisms occur in response to dopaminergic input in the rodent frontal cortex.

The present study shows that DA inhibits the SA of PTNs, through a non-selective or synergistic D1-D2 receptor mechanism. Additionally, DA reverses

glutamate induced excitation of SA in PTNs. Through these mechanisms, DA may have potent effects on PTN activity. Because PTNs are critical for the production of skilled voluntary movements. Dopamine's role in the motor cortex may be more important than previously thought.

Conclusions

The experiments in this dissertation have revealed various aspects of the dopaminergic circuitry and its function in the rodent motor cortex. Our observation of a widespread DA receptor distribution within the rodent motor cortex, suggests that DA affects the activity of a large proportion of motor cortex neurons. This broad target population indicates that DA has a potent influence over motor cortex activity, and therefore over motor behavior.

The diversity of receptor subtypes within one cortical region is surprising. Our findings indicate that numerous neurons contain D1a, D2, and D5 receptors. The unique laminar distributions of the receptor subtypes suggest that functionally distinct populations of neurons contain different subtypes. The DA receptor subfamilies are pharmacologically and structurally distinct, and therefore neuronal responses mediated by individual receptor subtypes are likely to differ. These features may endow a single neurotransmitter with a broad functional heterogeneity within the same cortical region.

This dissertation demonstrates that the distribution and morphology of neurons expressing DA receptors in the rodent motor cortex are similar to that

observed in primates. Therefore, studies from the rodent mesocortical dopaminergic system will be useful in understanding normal DA function in primates.

The differences in the mRNA and protein distribution for D5 receptors compared to the similarities in the mRNA and protein distribution for D1a and D2 receptors suggest a different turnover rate for different receptor subtypes. We also observed visible differences in the intracellular distribution of DA receptors. Both D1a and D2 receptor labeling were primarily visible in the somata and proximal dendrites of neurons, while D5 receptor label was clearly present in more distal portions of the apical dendrites. Additionally, extensive dendritic labeling for D1a receptors was observed in non-pyramidal neurons. This diverse cellular distribution of receptors suggests that intracellular receptor localization may be involved in differential responses to DA.

Our findings also demonstrate that DARPP-32 co-localizes with D1a, D2, and D5 receptors in neurons of rodent motor cortex. This suggests that DARPP-32 may be a more common phosphoprotein than originally believed. Pharmacological studies that examine the intracellular signal transduction mechanisms of all the DA receptors are needed to determine if DARPP-32 is indeed part of their signal transduction pathway.

Our results suggest three possible mechanisms by which DA is likely to exert complex modulatory influences over PTN activity. Our observation of the presence of three distinct DA receptor subtypes on PTNs, suggests that DA

directly modulates PTN activity through activation of one or more receptor subtypes. Our observation that DA reverses glutamate induced excitation of PTNs, suggests that DA may modulate PTN activity through a presynaptic interaction with glutamate terminals that may or may not be receptor mediated. We also observed D1a receptors in non-pyramidal neurons. Dopamine may indirectly affect PTN activity through activation of these interneurons. Any or all of these influences on PTNs will provide a direct link between the mesocortical dopaminergic system, and the control of skilled movements.

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